

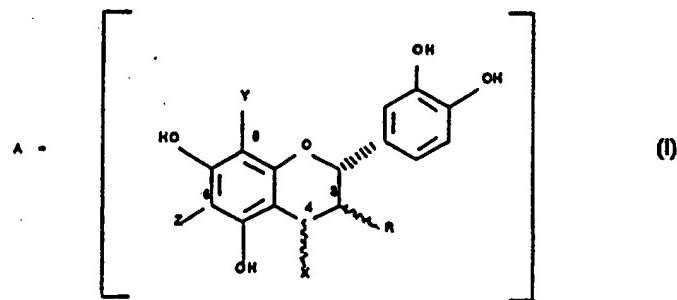
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(22) International Filing Date: 2 April 1996 (02.04.96)		
(71) Applicant (for all designated States except US): MARS, INCORPORATED [US/US]; 6885 Elm Street, McLean, VA 22101-3883 (US).		
(72) Inventors; and		Published
(75) Inventors/Applicants (for US only): ROMANCZYK, Leo, J., Jr. [US/US]; 4 Fern Drive R.D. #3, Hackettstown, NJ 07840 (US). HAMMERSTONE, John, F., Jr. [US/US]; 526 Fulmer Road, Nazareth, PA 18064 (US). BUCK, Margaret, M. [US/US]; 126 Lake Valley Road, Morristown, NJ 07960 (US). POST, Laurie, S. [US/US]; 17 Midland Drive, Great Meadows, NJ 07838 (US). CIPOLLA, Giovanni, G. [US/US]; 403 Third Avenue, Alpha, NJ 08865 (US). McCLELLAND, Craig, A. [US/US]; R.R. 5 Box 5621, East Stroudsburg, PA 18301 (US). MUNDT, Jeff, A. [US/US]; 7 Petersburg Road, Hackettstown, NJ 07840 (US). SCHMITZ, Harold, H. [US/US]; 61 Harlan School Road, Branchburg, NJ 08876 (US).	With international search report.	
(74) Agents: SANTISI, Leonard, J. et al.; Curtis, Morris & Safford, P.C., 530 Fifth Avenue, New York, NY 10036 (US).		

(54) Title: COCOA EXTRACT COMPOUNDS AND METHODS FOR MAKING AND USING THE SAME



(57) Abstract

Disclosed and claimed are cocoa extracts such as polyphenols or procyanidins, methods for preparing such extracts, as well as uses for them, especially a compound of formula (I), wherein: n is an integer from 3 to 12, such that there is a first monomeric unit A, and a plurality of other monomeric units; R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar; position 4 is alpha or beta stereochemistry; X, Y and Z represent positions for bonding between monomeric units, with the provisos that as to the first monomeric unit, bonding of another monomeric unit thereto is at position 4 and Y = Z = hydrogen, and, that when not for bonding monomeric units, X, Y and Z are hydrogen or alpha or beta sugar; and the sugar can be optionally substituted with a phenolic moiety via an ester bond.

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**COCOA EXTRACT COMPOUNDS AND METHODS
FOR MAKING AND USING THE SAME**

REFERENCE TO RELATED APPLICATION

5 Reference is made to copending U.S. application Serial No. 08/317,226, filed October 3, 1994, incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to cocoa extracts and 10 compounds therefrom such as polyphenols preferably polyphenols enriched with procyanidins. This invention also relates to methods for preparing such extracts and compounds, as well as to uses for them; for instance, as antineoplastic agents, antioxidants, DNA topoisomerase II 15 enzyme inhibitors, cyclo-oxygenase and/or lipoxygenase modulators, NO (Nitric Oxide) or NO-synthase modulators, blood or in vivo glucose modulators, and antimicrobials.

Documents are cited in this disclosure with a full 20 citation for each appearing thereat or in a References section at the end of the specification, preceding the claims. These documents pertain to the field of this invention; and, each document cited herein is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

25 Polyphenols are an incredibly diverse group of compounds (Ferreira et al., 1992) which widely occur in a variety of plants, some of which enter into the food chain. In some cases they represent an important class of compounds for the human diet. Although some of the polyphenols are 30 considered to be nonnutritive, interest in these compounds has arisen because of their possible beneficial effects on health. For instance, quercetin (a flavonoid) has been shown to possess anticarcinogenic activity in experimental

animal studies (Deshner et al., 1991 and Kato et al., 1983). (+)-Catechin and (-)-epicatechin (flavan-3-ols) have been shown to inhibit Leukemia virus reverse transcriptase activity (Chu et al., 1992). Nobotanin (an oligomeric hydrolyzable tannin) has also been shown to possess anti-tumor activity (Okuda et al., 1992). Statistical reports have also shown that stomach cancer mortality is significantly lower in the tea producing districts of Japan. Epigallocatechin gallate has been reported to be the pharmacologically active material in green tea that inhibits mouse skin tumors (Okuda et al., 1992). Ellagic acid has also been shown to possess anticarcinogen activity in various animal tumor models (Bukharta et al., 1992). Lastly, proanthocyanidin oligomers have been patented by the Kikkoman Corporation for use as antimutagens. Indeed, the area of phenolic compounds in foods and their modulation of tumor development in experimental animal models has been recently presented at the 202nd National Meeting of The American Chemical Society (Ho et al., 1992; Huang et al., 1992).

However, none of these reports teaches or suggests cocoa extracts or compounds therefrom, any methods for preparing such extracts or compounds therefrom, or, any uses for cocoa extracts or compounds therefrom, as antineoplastic agents, anti-oxidants, anti-microbials, cyclo-oxygenase and/or lipoxygenase modulators, NO or NO-synthase modulators, and blood or *in vivo* glucose modulators.

OBJECTS AND SUMMARY OF THE INVENTION

Since unfermented cocoa beans contain substantial levels of polyphenols, the present inventors considered it possible that similar activities of and uses for cocoa extracts, e.g., compounds within cocoa, could be revealed by

extracting such compounds from cocoa and screening the extracts for activity. The National Cancer Institute has screened various *Theobroma* and *Herrania* species for anti-cancer activity as part of their massive natural product selection program. Low levels of activity were reported in some extracts of cocoa tissues, and the work was not pursued. Thus, in the antineoplastic or anti-cancer art, cocoa and its extracts were not deemed to be useful; i.e., the teachings in the antineoplastic or anti-cancer art lead the skilled artisan away from employing cocoa and its extracts as cancer therapy.

Since a number of analytical procedures were developed to study the contributions of cocoa polyphenols to flavor development (Clapperton et al., 1992), the present inventors decided to apply analogous methods to prepare samples for anti-cancer screening, contrary to the knowledge in the antineoplastic or anti-cancer art. Surprisingly, and contrary to the knowledge in the art, e.g., the National Cancer Institute screening, the present inventors discovered that cocoa polyphenol extracts which contain procyanidins, have significant utility as anti-cancer or antineoplastic agents. Additionally, the inventors demonstrate that cocoa extracts containing procyanidins and compounds from cocoa extracts have utility as antioxidants, antineoplastics, antimicrobials, cyclo-oxygenase and/or lipoxygenase modulators, NO or NO-synthase modulators, and blood or *in vivo* glucose modulators.

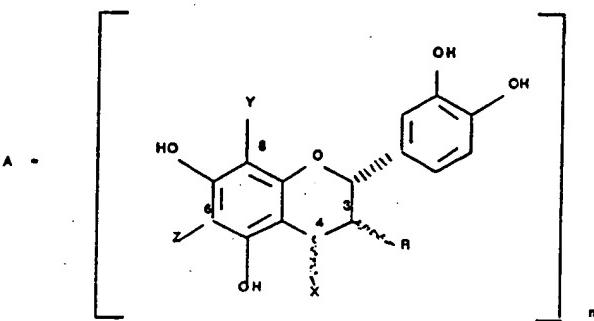
It is an object of the present invention to provide a method for producing cocoa extract and/or compounds therefrom.

It is another object of the invention to provide a cocoa extract and/or compounds therefrom.

It is still another object of the present invention to provide compounds of the formula and methods for obtaining a compound of the formula:

5

10



wherein:

15 n is an integer from 3 to 12, such that there is a first monomeric unit A, and a plurality of other monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

20 position 4 is alpha or beta stereochemistry;
 X, Y and Z represent positions for bonding between monomeric units, with the provisos that as to the first monomeric unit, bonding of another monomeric unit thereto is at position 4 and Y = Z = hydrogen, and, that when not for bonding monomeric units X, Y and Z are hydrogen, or Z, Y are sugar and X is hydrogen, or X is alpha or beta sugar and Z, Y are hydrogen, or combinations thereof. The compound can have n as 5 to 12, and certain preferred compounds have n as 5. The sugar can be selected from the group consisting
 25 essentially of glucose, galactose, xylose, rhamnose, and arabinose. The sugar of any or all of R, X, Y and Z can

optionally be substituted with a phenolic moiety via an ester bond.

It is another object of the invention to provide an antioxidant composition.

5 It is another object of the invention to demonstrate inhibition of DNA topoisomerase II enzyme activity.

It is yet another object of the present invention to provide a method for treating tumors or cancer.

10 It is still another object of the invention to provide an anti-cancer, anti-tumor or antineoplastic composition.

It is still a further object of the invention to provide an antimicrobial composition.

15 It is yet another object of the invention to provide a cyclo-oxygenase and/or lipoxygenase modulating composition.

It is still another object of the invention to provide an NO or NO-synthase-modulating composition.

20 It is another object of the invention to provide a blood or *in vivo* glucose-modulating composition.

It is yet a further object of the invention to provide a method for treating a patient with an antineoplastic, antioxidant, antimicrobial, cyclo-oxygenase 25 and/or lipoxygenase modulating or NO or NO-synthase modulating and/or blood or *in vivo* glucose-modulating composition.

It is a further object of the invention to provide a method for making any of the aforementioned compositions.

30 And, it is an object of the invention to provide a kit for use in the aforementioned methods or for preparing the aforementioned compositions.

It has been surprisingly discovered that cocoa extract, and compounds therefrom, have anti-tumor, anti-cancer or antineoplastic activity or, is an antioxidant composition or, inhibits DNA topoisomerase II enzyme 5 activity or, is an antimicrobial or, is a cyclo-oxygenase and/or lipoxygenase modulator or, is a NO or NO-synthase modulator or, is a blood or *in vivo* glucose modulator.

Accordingly, the present invention provides a substantially pure cocoa extract and compounds therefrom. 10 The extract or compounds preferably comprises polyphenol(s) such as polyphenol(s) enriched with cocoa procyanidin(s), such as polyphenols of at least one cocoa procyanidin selected from (-) epicatechin, (+) catechin, procyanidin B-2, procyanidin oligomers 2 through 12, preferably 2 through 15 5 or 4 through 12, more preferably 3 through 12, and most preferably 5 through 12, procyanidin B-5, procyanidin A-2 and procyanidin C-1.

The present invention also provides an anti-tumor, anti-cancer or antineoplastic or antioxidant or DNA 20 topoisomerase II inhibitor, or antimicrobial, or cyclo-oxygenase and/or lipoxygenase modulator, or an NO or NO-synthase modulator, or blood or *in vivo* glucose modulator composition comprising a substantially pure cocoa extract or compound therefrom or synthetic cocoa polyphenol(s) such as 25 polyphenol(s) enriched with procyanidin(s) and a suitable carrier, e.g., a pharmaceutically, veterinary or food science acceptable carrier. The extract or compound therefrom preferably comprises cocoa procyanidin(s). The cocoa extract or compounds therefrom is preferably obtained 30 by a process comprising reducing cocoa beans to powder, defatting the powder and, extracting and purifying active compound(s) from the powder.

The present invention further comprehends a method for treating a patient in need of treatment with an anti-tumor, anti-cancer, or antineoplastic agent or an antioxidant, or a DNA topoisomerase II inhibitor, or

5 antimicrobial, or cyclo-oxygenase and/or lipoxygenase modulator, or an NO or NO-synthase modulator, or blood or *in vivo* glucose modulator comprising administering to the patient a composition comprising an effective quantity of a substantially pure cocoa extract or compound therefrom or

10 synthetic cocoa polyphenol(s) or procyanidin(s) and a carrier, e.g., a pharmaceutically, veterinary or food science acceptable carrier. The cocoa extract or compound therefrom can be cocoa procyanidin(s); and, is preferably obtained by reducing cocoa beans to powder, defatting the

15 powder and, extracting and purifying active compound(s) from the powder.

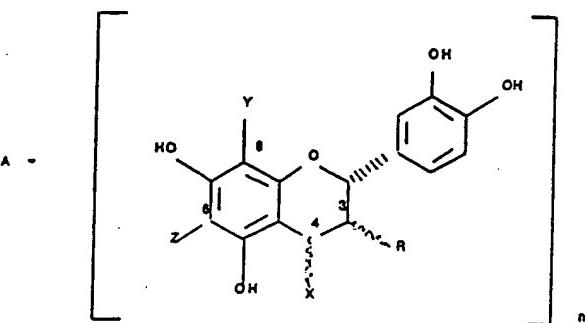
Additionally, the present invention provides a kit for treating a patient in need of treatment with an anti-tumor, anti-cancer, or antineoplastic agent or antioxidant or DNA topoisomerase II inhibitor, or antimicrobial, or cyclo-oxygenase and/or lipoxygenase modulator, or an NO or NO-synthase modulator, or blood or *in vivo* glucose modulator comprising a substantially pure cocoa extract or compounds therefrom or synthetic cocoa polyphenol(s) or procyanidin(s)

20 and a suitable carrier, e.g., a pharmaceutically, veterinary or food science acceptable carrier, for admixture with the extract or compound therefrom or synthetic polyphenol(s) or procyanidin(s).

Further, the present invention provides a compound

30 of the formula:

5



10 wherein:

n is an integer from 3 to 12, such that there is a first monomeric unit A, and a plurality of other monomeric units;

15 R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

position 4 is alpha or beta stereochemistry;

X, Y and Z represent positions for bonding between monomeric units, with the provisos that as to the first monomeric unit, bonding of another monomeric unit thereto is 20 at position 4 and Y = Z = hydrogen, and, that when not for bonding monomeric units, X, Y and Z are hydrogen, or Z, Y are sugar and X is hydrogen, or X is alpha or beta sugar and Z, Y are hydrogen, or combinations thereof. The compound can have n as 5 to 12, and certain preferred compounds have 25 n as 5. The sugar can be selected from the group consisting essentially of glucose, galactose, xylose, rhamnose, and arabinose. The sugar of any or all of R, X, Y and Z can optionally be substituted with a phenolic moiety via an ester bond. Preferred compounds are illustrated in Figs. 30 38A to 38P and 39A to 39AA. Linkages of 4-6 and 4-8 are presently preferred.

The present invention in another embodiment provides an antineoplastic composition comprising an inventive compound and a pharmaceutically, veterinary or food science acceptable carrier.

5 In a further embodiment the invention provides an antimicrobial composition comprising an inventive compound and a suitable carrier or diluent.

10 The invention also provides a cyclo-oxygenase and/or lipoxygenase modulator composition comprising an inventive compound and a suitable carrier or diluent.

The invention additionally provides a NO or NO-synthase-modulating composition comprising an inventive compound and a suitable carrier or diluent.

15 The invention comprehends a blood or *in vivo* glucose-modulating composition comprising an inventive compound and a suitable carrier or diluent.

20 Still further, the invention comprehends a method for treating a patient in need of treatment with an antineoplastic agent, or antioxidant agent/composition, or a DNA topoisomerase II inhibitor or composition, or a 25 antimicrobial agent/composition, or cyclo-oxygenase and/or lipoxygenase modulating agent/composition, or NO or NO-synthase modulating agent/composition, or blood or *in vivo* glucose-modulating agent/composition comprising administering to the patient a composition comprising an effective quantity of an inventive compound and a suitable carrier.

30 The invention even further encompasses food preservation or preparation compositions comprising an inventive compound, and methods for preparing or preserving food by adding the composition to food.

And, the invention still further encompasses a DNA topoisomerase II inhibitor comprising an inventive compound and a suitable carrier or diluent, and methods for treating a patient in need of such treatment by administration of the 5 composition.

Considering broadly the aforementioned embodiments involving cocoa extracts, the invention also includes such 10 embodiments wherein an inventive compound is used instead of or as the cocoa extracts. Thus, the invention comprehends kits, methods, and compositions analogous to those above-stated with regard to cocoa extracts and with an inventive 15 compound.

These and other objects and embodiments are disclosed or will be obvious from the following Detailed 15 Description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following Detailed Description will be better understood by reference to the accompanying drawings wherein:

20 Fig. 1 shows a representative gel permeation chromatogram from the fractionation of crude cocoa procyanidins;

25 Fig. 2A shows a representative reverse-phase HPLC chromatogram showing the separation (elution profile) of cocoa procyanidins extracted from unfermented cocoa;

Fig. 2B shows a representative normal phase HPLC separation of cocoa procyanidins extracted from unfermented cocoa;

30 Fig. 3 shows several representative procyanidin structures;

Figs. 4A-4E show representative HPLC chromatograms of five fractions employed in screening for anti-cancer or antineoplastic activity;

5 Figs. 5 and 6A-6D show the dose-response relationship between cocoa extracts and cancer cells ACHN (Fig. 5) and PC-3 (Figs. 6A-6D) (fractional survival vs. dose, $\mu\text{g/mL}$); M&M2 F4/92, M&MA+E U12P1, M&MB+E Y192P1, M&MC+E U12P2, M&MD+E U12P2;

10 Figs. 7A to 7H show the typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, A+B, A+E, and A+D, and the PC-3 cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1A 0212P3, MM-1 B 0162P1, MM-1 C 0122P3, MM-1 D 0122P3, MM-1 E 0292P8, MM-1 A/B 0292P6, MM-1 A/E 0292P6, MM-1 A/D 0292P6;

15 Figs. 8A to 8H show the typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, A+B, B+E, and D+E and the KB Nasopharyngeal/HeLa cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1A092K3, MM-1 B 0212K5, MM-1 C 0162K3, MM-1 D 0212K5, MM-1 E 0292K5, MM-1 A/B 0292K3, MM-1 B/E 0292K4, MM-1 D/E 0292K5;

20 Figs. 9A to 9H show the typical dose response relationship between cocoa procyanidin fractions A, B, C, D, E, B+D, A+E and D+E and the HCT-116 cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1 C 0192H5, D 0192H5, E 0192H5, MM-1 B&D 0262H2, A/E 0262H3, MM-1 D&E 0262H1;

25 Figs. 10A to 10H show typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, B+D, C+D and A+E and the ACHN renal cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1 A 092A5, MM-1 B 092A5, MM-1 C 0192A7, MM-1 D 0192A7, M&M1 E 0192A7, MM-1 B&D 0302A6, MM-1 C&D 0302A6, MM-1 A&E 0262A6;

Figs. 11A to 11H show typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, A+E, B+E and C+E and the A-549 lung cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1 A 019258, MM-1 B 5 09256, MM-1 C 019259, MM-1 D 019258, MM-1 E 019258, A/E 026254, MM-1 B&E 030255, MM-1 C&E N6255;

Figs. 12A to 12H show typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, B+C, C+D and D+E and the SK-5 melanoma cell line 10 (fractional survival vs. dose $\mu\text{g/mL}$); MM-1 A 0212S4, MM-1 B 0212S4, MM-1 C 0212S4, MM-1 D 0212S4, MM-1 E N32S1, MM-1 B&C N32S2, MM-1 C&D N32S3, MM-1 D&E N32S3;

Figs. 13A to 13H show typical dose response relationships between cocoa procyanidin fractions A, B, C, 15 D, E, B+C, C+E, and D+E and the MCF-7 breast cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1 A N22M4, MM-1 B N22M4, MM-1 C N22M4, MM-1 D N22M3, MM-1 E 0302M2, MM-1 B/C 0302M4, MM-1 C&E N22M3, MM-1 D&E N22M3;

Fig. 14 shows typical dose response relationships 20 for cocoa procyanidin (particularly fraction D) and the CCRF-CEM T-cell leukemia cell line (cells/mL vs. days of growth; open circle is control, darkened circle is 125 μg fraction D, open inverted triangle is 250 μg fraction D, darkened inverted triangle is 500 μg fraction D);

25 Fig. 15A shows a comparison of the XTT and Crystal Violet cytotoxicity assays against MCF-7 p168 breast cancer cells treated with fraction D+E (open circle is XTT and darkened circle is Crystal Violet);

Fig. 15B shows a typical dose response curve 30 obtained from MDA MB231 breast cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540nm) vs. Days; open circle is

control, darkened circle is vehicle, open inverted triangle is 250 μ g/mL, darkened inverted triangle is 100 μ g/mL, open square is 10 μ g/mL; absorbance of 2.0 is maximum of plate reader and may not be necessarily representative of cell number);

Fig. 15C shows a typical dose response curve obtained from PC-3 prostate cancer cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250 μ g/mL, darkened inverted triangle is 100 μ g/mL and open square is 10 μ g/mL);

Fig. 15D shows a typical dose-response curve obtained from MCF-7 p168 breast cancer cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250 μ g/mL, darkened inverted triangle is 100 μ g/mL, open square is 10 μ g/mL, darkened square is 1 μ g/mL; absorbance of 2.0 is maximum of plate reader and may not be necessarily representative of cell number);

Fig. 15E shows a typical dose response curve obtained from Hela cervical cancer cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250 μ g/mL, darkened inverted triangle is 100 μ g/mL, open square is 10 μ g/mL; absorbance of 2.0 is maximum of plate reader and may not be necessarily representative of cell number);

Fig. 15F shows cytotoxic effects against Hela cervical cancer cell line treated with different cocoa

polyphenol fractions (absorbance (540nm) vs. Days; open circle is 100 μ g/mL fractions A-E, darkened circle is 100 μ g/mL fractions A-C, open inverted triangle is 100 μ g/mL fractions D&E; absorbance of 2.0 is maximum of plate reader and not representative of cell number);

5 Fig. 15G shows cytotoxic effects at 100 μ l/mL against SKBR-3 breast cancer cell line treated with different cocoa polyphenol fractions (absorbance (540nm) vs. Days; open circle is fractions A-E, darkened circle is fractions A-C, open inverted triangle is fractions D&E);

10 Fig. 15H shows typical dose-response relationships between cocoa procyanidin fraction D+E on Hela cells (absorbance (540nm) vs. Days; open circle is control, darkened circle is 100 μ g/mL, open inverted triangle is 15 75 μ g/mL, darkened inverted triangle is 50 μ g/mL, open square is 25 μ g/mL, darkened square is 10 μ g/mL; absorbance of 2.0 is maximum of plate reader and is not representative of cell number);

15 Fig. 15I shows typical dose-response relationship between cocoa procyanidin fraction D+E on SKBR-3 cells (absorbance (540nm) vs. Days; open circle is control, darkened circle is 100 μ g/mL, open inverted triangle is 75 μ g/mL, darkened inverted triangle is 50 μ g/mL, open square is 25 μ g/mL, darkened square is 25 10 μ g/mL);

20 Fig. 15J shows typical dose-response relationships between cocoa procyanidin fraction D+E on Hela cells using the Soft Agar Cloning assay (bar chart; number of colonies vs. control, 1, 10, 50, and 30 100 μ g/mL);

25 Fig. 15K shows the growth inhibition of Hela cells when treated with crude polyphenol extracts obtained from

eight different cocoa genotypes (% control vs. concentration, $\mu\text{g/mL}$; open circle is C-1, darkened circle is C-2, open inverted triangle is C-3, darkened inverted triangle is C-4, open square is C-5, darkened square is C-6, 5 open triangle is C-7, darkened triangle is C-8; C-1 = UF-12: horti race = Trinitario and description is crude extracts of UF-12 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-2 = NA-33: horti race = Forastero and description is crude extracts of NA-33 10 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-3 = EEG-48: horti race = Forastero and description is crude extracts of EEG-48 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-4 = unknown: horti race = Forastero and description is crude extracts of 15 unknown (W. African) cocoa polyphenols (decaffeinated/detheobrominated); C-5 = UF-613: horti race = Trinitario and description is crude extracts of UF-613 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-6 = ICS-100: horti race = Trinitario 20 (to Nicaraguan Criollo ancestor) and description is crude extracts of ICS-100 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-7 = ICS-139: horti race = Trinitario (Nicaraguan Criollo ancestor) and description is crude extracts of ICS-139 (Brazil) cocoa polyphenols 25 (decaffeinated/detheobrominated); C-8 = UIT-1: horti race = Trinitario and description is crude extracts of UIT-1 (Malaysia) cocoa polyphenols (decaffeinated/detheobrominated);

Fig. 15L shows the growth inhibition of Hela cells 30 when treated with crude polyphenol extracts obtained from fermented cocoa beans and dried cocoa beans (stages throughout fermentation and sun drying; % control vs.

concentration, $\mu\text{g/mL}$; open circle is day zero fraction, darkened circle is day 1 fraction, open inverted triangle is day 2 fraction, darkened inverted triangle is day 3 fraction, open square is day 4 fraction and darkened square is day 9 fraction);

Fig. 15M shows the effect of enzymatically oxidized cocoa procyanidins against Hela cells (dose response for polyphenol oxidase treated crude cocoa polyphenol; % control vs. concentration, $\mu\text{g/mL}$; darkened square is crude UIT-1 (with caffeine and theobromine), open circle crude UIT-1 (without caffeine and theobromine) and darkened circle is crude UIT-1 (polyphenol oxidase catalyzed);

Fig. 15N shows a representative semi-preparative reverse phase HPLC separation for combined cocoa procyanidin fractions D and E;

Fig. 15O shows a representative normal phase semi-preparative HPLC separation of a crude cocoa polyphenol extract;

Fig. 16 shows typical Rancimat Oxidation curves for cocoa procyanidin extract and fractions in comparison to the synthetic antioxidants BHA and BHT (arbitrary units vs. time; dotted line and cross (+) is BHA and BHT; * is D-E; x is crude; open square is A-C; and open diamond is control);

Fig. 17 shows a typical Agarose Gel indicating inhibition of topoisomerase II catalyzed decatenation of kinetoplast DNA by cocoa procyanidin fractions (Lane 1 contains 0.5 μg of marker (M) monomer-length kinetoplast DNA circles; Lanes 2 and 20 contain kinetoplast DNA that was incubated with Topoisomerase II in the presence of 4% DMSO, but in the absence of any cocoa procyanidins. (Control -C); Lanes 3 and 4 contain kinetoplast DNA that was incubated

with Topoisomerase II in the presence of 0.5 and 5.0 μ g/mL cocoa procyanidin fraction A; Lanes 5 and 6 contain kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.5 and 5.0 μ g/mL cocoa procyanidin fraction 5 B; Lanes 7, 8, 9, 13, 14 and 15 are replicates of kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.05, 0.5 and 5.0 μ g/mL cocoa procyanidin fraction D; Lanes 10, 11, 12, 16, 17 and 18 are replicates 10 of kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.05, 0.5, and 5.0 μ g/mL cocoa procyanidin fraction E; Lane 19 is a replicate of kinetoplast DNA that was incubated with Topoisomerase II in the presence of 5.0 μ g/mL cocoa procyanidin fraction E);

Fig. 18 shows dose response relationships of cocoa 15 procyanidin fraction D against DNA repair competent and deficient cell lines (fractional survival vs. μ g/mL; left side xrs-6 DNA Deficient Repair Cell Line, MM-1 D D282X1; right side BR1 Competent DNA Repair Cell Line, MM-1 D D282B1);

Fig. 19 shows the dose-response curves for 20 Adriamycin resistant MCF-7 cells in comparison to a MCF-7 p168 parental cell line when treated with cocoa fraction D+E (% control vs. concentration, μ g/mL; open circle is MCF-7 p168; darkened circle is MCF-7 ADR);

Figs. 20A and B show the dose-response effects on 25 Hela and SKBR-3 cells when treated at 100 μ g/mL and 25 μ g/mL levels of twelve fractions prepared by Normal phase semi-preparative HPLC (bar chart, % control vs. control and fractions 1-12);

Fig. 21 shows a normal phase HPLC separation of 30 crude, enriched and purified pentamers from cocoa extract;

Figs. 22A, B and C show MALDI-TOF/MS of pentamer enriched procyanidins, and of Fractions A-C and of Fractions D-E, respectively;

Fig. 23A shows an elution profile of oligomeric 5 procyanidins purified by modified semi-preparative HPLC;

Fig. 23B shows an elution profile of a trimer procyanidin by modified semi-preparative HPLC;

Figs. 24A-D each show energy minimized structures 10 of all (4-8) linked pentamers based on the structure of epicatechin;

Fig. 25A shows relative fluorescence of epicatechin upon thiolysis with benzylmercapten;

Fig. 25B shows relative fluorescence of catechin 15 upon thiolysis with benzylmercapten;

Fig. 25C shows relative fluorescence of dimers (B2 and B5) upon thiolysis with benzylmercapten;

Fig. 26A shows relative fluorescence of dimer upon thiolysis;

Fig. 26B shows relative fluorescence of B5 dimer 20 upon thiolysis of dimer and subsequent desulphurization;

Fig. 27A shows the relative tumor volume during treatment of MDA MB 231 nude mouse model treated with pentamer;

Fig. 27B shows the relative survival curve of 25 pentamer treated MDA 231 nude mouse model;

Fig. 28 shows the elution profile from halogen-free analytical separation of acetone extract of procyanidins from cocoa extract;

Fig. 29 shows the effect of pore size of 30 stationary phase for normal phase HPLC separation of procyanidins;

Fig. 30A shows the substrate utilization during fermentation of cocoa beans;

Fig. 30B shows the metabolite production during fermentation;

5 Fig. 30C shows the plate counts during fermentation of cocoa beans;

Fig. 30D shows the relative concentrations of each component in fermented solutions of cocoa beans;

10 Fig. 31 shows the acetylcholine-induced relaxation of NO-related phenylephrine-precontracted rat aorta;

Fig. 32 shows the blood glucose tolerance profiles from various test mixtures;

Figs. 33A-B show the effects of indomethacin on COX-1 and COX-2 activities;

15 Figs. 34A-B show the correlation between the degree of polymerization and IC_{50} vs. COX-1/COX-2 (μM);

Fig. 35 shows the correlation between the effects of compounds on COX-1 and COX-2 activities expressed as μM ;

20 Figs. 36A-V show the IC_{50} values (μM) of samples containing procyanidins with COX-1/COX-2;

Fig. 37 shows the purification scheme for the isolation of procyanidins from cocoa;

Fig. 38A to 38P shows the preferred structures of the pentamer;

25 Figs. 39A-AA show a library of stereoisomers of pentamers;

Figs. 40A-B show 70 minute gradients for normal phase HPLC separation of procyanidins, detected by UV and fluorescence, respectively;

30 Figs. 41A-B show 30 minute gradients for normal phase HPLC separation of procyanidins, detected by UV and fluorescence, respectively;

Fig. 42 shows a preparation normal phase HPLC separation of procyanidins;

Figs. 43A-G show CD (circular dichroism) spectra of procyanidin dimers, trimers, tetramers, pentamers, 5 hexamers, heptamers and octamers, respectively;

Fig. 44A shows the structure and $^1\text{H}/^{13}\text{C}$ NMR data for epicatechin;

Figs. 44B-F show the APT, COSY, XHCORR, ^1H and ^{13}C NMR spectra for epicatechin;

10 Fig. 45A shows the structure and $^1\text{H}/^{13}\text{C}$ NMR data for catechin;

Figs. 45B-E show the ^1H , APT, XHCORR and COSY NMR spectra for catechin;

15 Fig. 46A shows the structure and $^1\text{H}/^{13}\text{C}$ NMR data for B2 dimer;

Figs. 46B-G show the ^{13}C , APT, ^1H , HMQC, COSY and HOHAHA NMR spectra for the B2 dimer;

Fig. 47A shows the structure and $^1\text{H}/^{13}\text{C}$ NMR data for B5 dimer;

20 Figs. 47B-G show the ^1H , ^{13}C , APT, COSY, HMQC and HOHAHA NMR spectra for B5 dimer;

Figs. 48A-D show the ^1H , COSY, HMQC and HOHAHA NMR spectra for epicatechin/catechin trimer; and

25 Figs. 49A-D show the ^1H , COSY, HMQC and HOHAHA NMR spectra for epicatechin trimer.

DETAILED DESCRIPTION

As discussed above, it has now been surprisingly found that cocoa extracts or a compound therefrom exhibit anti-cancer, anti-tumor or antineoplastic activity, 30 antioxidant activity, inhibit DNA topoisomerase II enzyme and have antimicrobial, cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase and blood or *in vivo* glucose modulating

activities. The extracts or compound therefrom are generally prepared by reducing cocoa beans to a powder, defatting the powder, and extracting and purifying the active compound(s) from the defatted powder. The powder can 5 be prepared by freeze-drying the cocoa beans and pulp, depulping and dehulling the freeze-dried cocoa beans and grinding the dehulled beans. The extraction of active compound(s) can be by solvent extraction techniques. The extracts can be purified; for instance, by gel permeation 10 chromatography or by preparative High Performance Liquid Chromatography (HPLC) techniques or by a combination of such techniques. An outline of the purification protocol utilized in the isolation of substantially pure procyanidins is shown in Fig. 37. Steps 1 and 2 of the purification 15 scheme are described in Examples 1 and 2; steps 3 and 4 are described in Examples 3, 13 and 23; step 5 is described in Examples 4 and 14; and step 6 is described in Examples 4, 14 and 16. The skilled artisan would appreciate and envision modifications in the purification scheme outlined in Figure 20 37 to obtain the active compounds without departing from the spirit or scope thereof and without undue experimentation. The extracts or compound therefrom having activity, without wishing to necessarily be bound by any particular theory, have been identified as cocoa polyphenol(s) such as 25 procyanidins. These cocoa procyanidins have significant anti-cancer, anti-tumor or antineoplastic activity; antioxidant activity; and inhibit DNA topoisomerase II enzyme; possess antimicrobial activity; and can modulate cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase, and 30 blood or *in vivo* glucose.

As recited above, the invention involves certain inventive compounds displaying the utilities noted above for

cocoa extracts; and, throughout this disclosure, the term "cocoa extract" may be substituted by an inventive compound disclosed above, such that it is to be understood that an inventive compound can be the cocoa extract. Preferred 5 inventive compounds are shown in Fig. 38A to 38P, and Fig. 39A to 39V show a library of stereoisomers of the pentamer from which other compounds within the scope of the invention may be obtained without undue experimentation.

Anti-cancer, anti-tumor or antineoplastic or, 10 antioxidant, DNA topoisomerase II enzyme inhibiting, antimicrobial, cyclo-oxygenase and/or lipoxygenase modulator NO- or NO-synthase and blood or in vivo glucose modulating activities, or compositions containing the inventive cocoa polyphenols or procyanidins can be prepared in accordance 15 with standard techniques well known to those skilled in the pharmaceutical or food science or veterinary art(s).

Such compositions can be administered to a subject or patient in need of such administration in dosages and by techniques well known to those skilled in the medical, 20 nutritional or veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular subject or patient, and the route of administration. The compositions can be co-administered or sequentially administered with other antineoplastic, anti-tumor or anti-cancer agents, antioxidants, DNA topoisomerase 25 II enzyme inhibiting agents, or cyclo-oxygenase and/or lipoxygenase, blood or in vivo glucose or NO or NO-synthase modulating agents and/or with agents which reduce or alleviate ill effects of antineoplastic, anti-tumor, anti- 30 cancer agents, antioxidants, DNA topoisomerase II enzyme inhibiting agents, cyclo-oxygenase and/or lipoxygenase, blood or in vivo glucose or NO or NO-synthase modulating

agents; again, taking into consideration such factors as the age, sex, weight, and condition of the particular subject or patient, and, the route of administration.

Examples of compositions of the invention include
5 edible compositions for oral administration such solid or liquid formulations, for instance, capsules, tablets, pills and the like, as well as chewable solid or beverage formulations, to which the present invention may be well-suited since it is from an edible source (e.g., cocoa or
10 chocolate flavored solid or liquid compositions); liquid preparations for orifice, e.g., oral, nasal, anal, vaginal etc., administration such as suspensions, syrups or elixirs (including cocoa or chocolate flavored compositions); and, preparations for parental, subcutaneous, intradermal,
15 intramuscular or intravenous administration (e.g., injectable administration) such as sterile suspensions or emulsions. However, the active ingredient in the compositions may complex with proteins such that when administered into the bloodstream, clotting may occur due to
20 precipitation of blood proteins; and, the skilled artisan should take this into account. In such compositions the active cocoa extract may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, DMSO, ethanol, or the like.
25 The active cocoa extract of the invention can be provided in lyophilized form for reconstituting, for instance, in isotonic aqueous, saline, glucose or DMSO buffer. In certain saline solutions, some precipitation has been observed; and, this observation may be employed as a means
30 to isolate inventive compounds, e.g., by a "salting out" procedure.

Further, the invention also comprehends a kit wherein the active cocoa extract is provided. The kit can include a separate container containing a suitable carrier, diluent or excipient. The kit can also include an 5 additional anti-cancer, anti-tumor or antineoplastic agent, antioxidant, DNA topoisomerase II enzyme inhibitor or antimicrobial, or cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase or blood or *in vivo* glucose modulating agent and/or an agent which reduces or alleviates ill effects of 10 antineoplastic, anti-tumor or anti-cancer agents, antioxidant, DNA topoisomerase II enzyme inhibitor or antimicrobial, or cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase and blood or *in vivo* glucose modulating agents for co- or sequential-administration. The additional 15 agent(s) can be provided in separate container(s) or in admixture with the active cocoa extract. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration.

Furthermore, while the invention is described with 20 respect to cocoa extracts preferably comprising cocoa procyanidins, from this disclosure the skilled organic chemist will appreciate and envision synthetic routes to obtain the active compounds. Accordingly, the invention comprehends synthetic cocoa polyphenols or procyanidins or 25 their derivatives which include, but are not limited to glycosides, gallates, esters, etc. and the like. That is, the inventive compounds can be prepared from isolation from cocoa or from any species within the *Theobroma* or *Herrania* genera, as well as from synthetic routes; and derivatives of 30 the inventive compounds such as glycosides, gallates, esters, etc. are included in the inventive compounds. Also, with reference to isolation from cocoa, any species of

Theobroma or Herrania or their inter- and intra- specific crosses thereof may be employed therefor, and reference in this regard is made to Schultes, "Synopsis of Herrania," Journal of the Arnold Arboretum, Vol. XXXIX, pp. 217 to 278 plus plates I to XVII (1958), Cuatrecasas, "Cacao and its Allies A Taxonomic Revision of the Genus Theobroma," Bulletin of the United States National Museum, Vol. 35, part 6, pp. 379 to 613, plus plates 1 to 11 (Smithsonian Institution 1964), and Addison et al., "Observations on the Species of the Genus Theobroma Which Occurs in the Amazon," Bol. Tech. Inst. Agronomico do Nortes, 25, 3 (1951).

The following non-limiting Examples are given by way of illustration only and are not to be considered a limitation of this invention, many apparent variations of which are possible without departing from the spirit or scope thereof.

EXAMPLES

Example 1: Cocoa Source and Method of Preparation

Several Theobroma cacao genotypes which represent the three recognized horticultural races of cocoa (Enriquez, 1967; Engels, 1981) were obtained from the three major cocoa producing origins of the world. A list of those genotypes used in this study are shown in Table 1. Harvested cocoa pods were opened and the beans with pulp were removed for freeze drying. The pulp was manually removed from the freeze dried mass and the beans were subjected to analysis as follows. The unfermented, freeze dried cocoa beans were first manually dehulled, and ground to a fine powdery mass with a TEKMAR Mill. The resultant mass was then defatted overnight by Soxhlet extraction using redistilled hexane as the solvent. Residual solvent was removed from the defatted mass by vacuum at ambient temperature.

Table 1: Description of *Theobroma cacao* Source Material

	GENOTYPE	ORIGIN	HORTICULTURAL RACE
5	UIT-1	Malaysia	Trinitario
	Unknown	West Africa	Forastero
	ICS-100	Brazil	Trinitario (Nicaraguan Criollo ancestor)
	ICS-39	Brazil	Trinitario (Nicaraguan Criollo ancestor)
10	UF-613	Brazil	Trinitario
	EEG-48	Brazil	Forastero
	UF-12	Brazil	Trinitario
	NA-33	Brazil	Forastero

Example 2: Procyanidin Extraction ProceduresA. Method 1

15 Procyanidins were extracted from the defatted, unfermented, freeze dried cocoa beans of Example 1 using a modification of the method described by Jalal and Collin (1977). Procyanidins were extracted from 50 gram batches of the defatted cocoa mass with 2X 400 mL 70% acetone/deionized water followed by 400mL 70% methanol/deionized water. The extracts were pooled and the solvents removed by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was diluted to 1L with deionized water and extracted 2X with 400mL CHCl₃. The solvent phase 20 was discarded. The aqueous phase was then extracted 4X with 500mL ethyl acetate. Any resultant emulsions were broken by 25

centrifugation on a Sorvall RC 28S centrifuge operated at 2,000 xg for 30 min. at 10°C. To the combined ethyl acetate extracts, 100-200mL deionized water was added. The solvent was removed by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was frozen in liquid N₂ followed by freeze drying on a LABCONCO Freeze Dry System. The yields of crude procyanidins that were obtained from the different cocoa genotypes are listed in Table 2.

10 **Table 2: Crude Procyanidin Yields**

GENOTYPE	ORIGIN	YIELDS (g)
UIT-1	Malaysia	3.81
Unknown	West Africa	2.55
ICS-100	Brazil	3.42
ICS-39	Brazil	3.45
UF-613	Brazil	2.98
EEG-48	Brazil	3.15
UF-12	Brazil	1.21
NA-33	Brazil	2.23

20

B. Method 2

Alternatively, procyanidins are extracted from defatted, unfermented, freeze dried cocoa beans of Example 1 with 70% aqueous acetone. Ten grams of defatted material 25 was slurried with 100 mL solvent for 5-10 min. The slurry was centrifuged for 15 min. at 4°C at 3000 xg and the supernatant passed through glass wool. The filtrate was subjected to distillation under partial vacuum and the resultant aqueous phase frozen in liquid N₂, followed by

freeze drying on a LABCONCO Freeze Dry System. The yields of crude procyanidins ranged from 15-20%.

Without wishing to be bound by any particular theory, it is believed that the differences in crude yields reflected variations encountered with different genotypes, geographical origin, horticultural race, and method of preparation.

Example 3: Partial Purification of Cocoa Procyanidins

A. Gel Permeation Chromatography

10 Procyanidins obtained from Example 2 were partially purified by liquid chromatography on Sephadex LH-20 (28 x 2.5 cm). Separations were aided by a step gradient from deionized water into methanol. The initial gradient composition started with 15% methanol in deionized water
15 which was followed step wise every 30 min. with 25% methanol in deionized water, 35% methanol in deionized water, 70% methanol in deionized water, and finally 100% methanol. The effluent following the elution of the xanthine alkaloids (caffeine and theobromine) was collected as a single
20 fraction. The fraction yielded a xanthine alkaloid free subfraction which was submitted to further subfractionation to yield five subfractions designated MM2A through MM2E. The solvent was removed from each subfraction by evaporation at 45°C with a rotary evaporator held under partial vacuum.
25 The resultant aqueous phase was frozen in liquid N₂ and freeze dried overnight on a LABCONCO Freeze Dry System. A representative gel permeation chromatogram showing the fractionation is shown in Figure 1. Approximately, 100mg of material was subfractionated in this manner.
30 Figure 1: Gel Permeation Chromatogram of Crude Procyanidins on Sephadex LH-20

Chromatographic Conditions: Column; 28 x 2.5 cm Sephadex LH-20, Mobile Phase: Methanol/Water Step Gradient, 15:85, 25:75, 35:65, 70:30, 100:0 Stepped at 1/2 Hour Intervals, Flow Rate; 1.5mL/min, Detector; UV at λ_1 = 254 nm and λ_2 = 5 365 nm, Chart Speed: 0.5mm/min, Column Load; 120mg.

B. Semi-preparative High Performance Liquid Chromatography (HPLC)

Method 1. Reverse Phase Separation

10 Procyanidins obtained from Example 2 and/or 3A were partially purified by semi-preparative HPLC. A Hewlett Packard 1050 HPLC System equipped with a variable wavelength detector, Rheodyne 7010 injection valve with
15 1mL injection loop was assembled with a Pharmacia FRAC-100 Fraction Collector. Separations were effected on a Phenomenex Ultracarb™ 10 μ ODS column (250 x 22.5mm) connected with a Phenomenex 10 μ ODS Ultracarb™ (60 x 10 mm) guard column. The mobile phase composition was
20 A = water; B = methanol used under the following linear gradient conditions: [Time, %A]; (0,85), (60,50), (90,0), and (110,0) at a flow rate of 5mL/min. Compounds were detected by UV at 254nm

A representative Semi-preparative HPLC trace is
25 shown in Figure 15N for the separation of procyanidins present in fraction D + E. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further purification and subsequent evaluation. Injection loads ranged from 25-100mg
30 of material.

Method 2. Normal Phase Separation

Procyanidin extracts obtained from Examples 2 and/or 3A were partially purified by semi-preparative HPLC. A Hewlett Packard 1050 HPLC system, Millipore-Waters

Model 480 LC detector set at 254nm was assembled with a Pharmacia Frac-100 Fraction Collector set in peak mode. Separations were effected on a Supelco 5 μ m Supelcosil LC-Si column (250 x 10mm) connected with a Supelco 5 μ m Supelguard LC-Si guard column (20 x 4.6mm). Procyanidins were eluted by a linear gradient under the following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86) followed by a 10 min. re-equilibration. Mobile phase composition was A = dichloromethane; B = methanol; and C = acetic acid: water (1:1). A flow rate of 3mL/min was used. Components were detected by UV at 254nm, and recorded on a Kipp & Zonen BD41 recorder. Injection volumes ranged from 100-250 μ l of 10mg of procyanidin extracts dissolved in 0.25mL 70% aqueous acetone. A representative semi-preparative HPLC trace is shown in Figure 15 O. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further purification and subsequent evaluation.

HPLC Conditions: 250 x 10mm Supelco Supelcosil LC-Si
(5 μ m) Semipreparative Column
20 x 4.6mm Supelco Supelcosil LC-Si
(5 μ m) Guard Column

5

Detector: Waters LC
Spectrophotometer Model
480 \AA 254nm

10

Flow rate: 3mL/min,
Column Temperature: ambient,
Injection: 250 μ L of 70% aqueous
acetone extract.

15	Gradient: Time (min)	CH ₂ Cl ₂	Methanol	Acetic Acid:H ₂ O (1:1)
0	82	14	4	
30	67.6	28.4	4	
60	46	50	4	
65	10	86	4	
20	70	10	86	4

The fractions obtained were as follows:

FRACTION	TYPE
1	dimers
2	trimers
3	tetramers
4	pentamers
5	hexamers
6	heptamers
7	octamers
10	nonamers
8	decamers
9	undecamers
11	dodecamers
12	higher oligomers

15

Example 4: Analytical HPLC Analysis of Procyanidin Extracts

Method 1. Reverse Phase Separation

20 Procyanidin extracts obtained from Example 3 were filtered through a 0.45μ filter and analyzed by a Hewlett Packard 1090 ternary HPLC system equipped with a Diode Array detector and a HP model 1046A Programmable Fluorescence Detector. Separations were effected at 45°C on a Hewlett-
 25 Packard 5μ Hypersil ODS column ($200 \times 2.1\text{mm}$). The flavanols and procyanidins were eluted with a linear gradient of 60% B into A followed by a column wash with B at a flow rate of 0.3mL/min . The mobile phase composition was B = 0.5% acetic acid in methanol and A = 0.5% acetic acid in nanopure water.

Acetic acid levels in A and B mobile phases can be increased to 2%. Components were detected by fluorescence, where

- $\lambda_{ex} = 276\text{nm}$ and $\lambda_{em} = 316\text{nm}$ and by UV at 280nm.
- Concentrations of (+)-catechin and (-)-epicatechin were
- 5 determined relative to reference standard solutions.
- Procyanidin levels were estimated by using the response factor for (-)-epicatechin. A representative HPLC chromatogram showing the separation of the various components is shown in Figure 2A for one cocoa genotype.
- 10 Similar HPLC profiles were obtained from the other cocoa genotypes.

HPLC Conditions: Column: 200 x 2.1mm Hewlett Packard Hypersil ODS (5μ)
 Guard column: 20 x 2.1mm Hewlett
 15 Packard Hypersil ODS (5μ)
 Detectors: Diode Array @ 280nm
 Fluorescence $\lambda_{ex} = 276\text{nm}$;
 $\lambda_{em} = 316\text{nm}$.
 Flow rate: 0.3mL/min.
 20 Column Temperature: 45°C

Gradient: Time (min)	0.5% Acetic Acid in nanopure water	0.5% Acetic acid in methanol
0	100	0
25 50	40	60
60	0	100

Method 2. Normal Phase Separation

Procyanidin extracts obtained from Examples 2
 30 and/or 3 were filtered through a 0.45μ filter and analyzed
 by a Hewlett Packard 1090 Series II HPLC system equipped

with a HP model 1046A Programmable Fluorescence detector and Diode Array detector. Separations were effected at 37°C on a 5 μ Phenomenex Lichrosphere® Silica 100 column (250 x 3.2mm) connected to a Supelco Supelguard LC-Si 5 μ guard column (20 x 4.6mm). Procyanidins were eluted by linear gradient under the following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86) followed by an 8 min. re-equilibration. Mobile phase composition was A=dichloromethane, B=methanol, and C=acetic acid: water at a volume ratio of 1:1. A flow rate of 0.5 mL/min. was used. Components were detected by fluorescence, where λ_{ex} = 276nm and λ_{em} = 316nm or by UV at 280 nm. A representative HPLC chromatogram showing the separation of the various procyanidins is shown in Figure 2B for one genotype. Similar HPLC profiles were obtained from other cocoa genotypes.

HPLC Conditions:

20 250 x 3.2mm Phenomenex Lichrosphere® Silica 100 column (5 μ) 20 x 4.6mm Supelco Supelguard LC-Si (5 μ) guard column
 Detectors: Photodiode Array @ 280nm
 Fluorescence λ_{ex} = 276nm;
 λ_{em} = 316nm.
 Flow rate: 0.5 mL/min.
 Column Temperature: 37°C

Gradient: Time (min.)	CH ₂ -Cl ₂	Methanol	Acetic Acid/Water (1:1)
0	82	14	4
30	67.6	28.4	4
60	46	50	4
65	10	86	4
70	10	86	4

Example 5:Identification of Procyanidins

Procyanidins were purified by liquid chromatography on Sephadex LH-20 (28 x 2.5cm) columns followed by semi-preparative HPLC using a 10 μ Bondapak C18 5 (100 x 8mm) column or by semi-preparative HPLC using a 5 μ Supelcosil LC-Si (250 x 10mm) column.

- Partially purified isolates were analyzed by Fast Atom Bombardment - Mass Spectrometry (FAB-MS) on a VG ZAB-T high resolution MS system using a Liquid Secondary Ion Mass 10 Spectrometry (LSIMS) technique in positive and negative ion modes. A cesium ion gun was used as the ionizing source at 30kV and a "Magic Bullet Matrix" (1:1 dithiothreitol/dithioerythritol) was used as the proton donor.
- 15 Analytical investigations of these fractions by LSIMS revealed the presence of a number of flavan-3-ol oligomers as shown in Table 3.

Table 3: LSIMS (Positive Ion) Data from Cocoa Procyanidin Fractions

	Oligomer	(M + 1) <i>m/z</i>	(M + Na) <i>m/z</i>	Mol. Wt.
5	Monomers (catechins)	291	313	290
	Dimer(s)	577/579	599/601	576/578
	Trimer(s)	865/867	887/889	864/866
	Tetramer(s)	1155	1177	1154
10	Pentamer(s)	1443	1465	1442
	Hexamer(s)	1731	1753	1730
	Heptamer(s)	---	2041	2018
	Octamer(s)	---	2329	2306
15	Nonamer(s)	---	2617	2594
	Decamer(s)	---	2905	2882
	Undecamer(s)	---	---	3170
	Dodecamer(s)	---	---	3458

The major mass fragment ions were consistent with work previously reported for both positive and negative ion FAB-MS analysis of procyanidins (Self et al., 1986 and Porter et al., 1991). The ion corresponding to *m/z* 577 ($M+H$)⁺ and its sodium adduct at *m/z* 599 ($M+Na$)⁺ suggested the presence of doubly linked procyanidin dimers in the isolates. It was interesting to note that the higher oligomers were more likely to form sodium adducts ($M+Na$)⁺ than their protonated molecular ions ($M+H$)⁺. The procyanidin isomers B-2, B-5 and C-1 were tentatively identified based on the work reported by Revilla et al. (1991), Self et al. (1986) and Porter et al. (1991). Procyanidins up to both the octamer and decamer were

verified by FAB-MS in the partially purified fractions. Additionally, evidence for procyanidins up to the dodecamer were observed from normal phase HPLC analysis (see Figure 2B). Without wishing to be bound by any particular theory, it is believed that the dodecamer is the limit of solubility in the solvents used in the extraction and purification schemes. Table 4 lists the relative concentrations of the procyanidins found in xanthine alkaloid free isolates based on reverse phase HPLC analysis.

Table 5 lists the relative concentrations of the procyanidins based on normal phase HPLC analysis.

Table 4: Relative Concentrations of Procyanidins in the Xanthine Alkaloid Free Isolates

15	Component	Amount
	(+)-catechin	1.6%
	(-)-epicatechin	38.2%
20	B-2 Dimer	11.0%
	B-5 Dimer	5.3%
	C-1 Trimer	9.3%
	Doubly linked dimers	3.0%
	Tetramer(s)	4.5%
25	Pentamer-Octamer	24.5%
	Unknowns and higher oligomers	2.6%

Table 5: Relative Concentrations of Procyanidins in Aqueous Acetone Extracts

	Component	Amount
5	(+)-catechin and (-) -epicatechin	41.9%
	B-2 and B-5 Dimers	13.9%
	Trimers	11.3%
	Tetramers	9.9%
10	Pentamers	7.8%
	Hexamers	5.1%
	Heptamers	4.2%
	Octamers	2.8%
	Nonamers	1.6%
15	Decamers	0.7%
	Undecamers	0.2%
	Dodecamers	<0.1%

Figure 3 shows several procyanoindin structures and
 20 Figures 4A-4E show the representative HPLC chromatograms of the five fractions employed in the following screening for anti-cancer or antineoplastic activity. The HPLC conditions for Figs. 4A-4E were as follows:

HPLC Conditions: Hewlett Packard 1090 ternary
 25 HPLC System equipped with HP Model 1046A Programmable Fluorescence Detector.
 Column: Hewlett Packard 5 μ Hypersil ODS (200 x 2.1mm) Linear Gradient of 60% B into A at a flow rate of 0.3mL/min. B = 0.5% acetic acid in methanol; A = 0.5% acetic acid in deionized water. $\lambda_{ex} = 280\text{nm}$; $\lambda_{em} = 316\text{nm}$.

Figure 15 O shows a representative semi-prep HPLC chromatogram of an additional 12 fractions employed in the screening for anticancer or antineoplastic activity (HPLC conditions stated above).

5 **Example 6: Anti-Cancer, Anti-Tumor or Antineoplastic Activity of Cocoa Extracts (Procyanidins)**

The MTT (3-[4,5-dimethyl thiazol-2yl]-2,5-diphenyltetrazolium bromide) - microtiter plate tetrazolium cytotoxicity assay originally developed by Mosmann (1983) was used to screen test samples from Example 5. Test samples, standards (cisplatin and chlorambucil) and MTT reagent were dissolved in 100% DMSO (dimethyl sulfoxide) at a 10mg/mL concentration. Serial dilutions were prepared from the stock solutions. In the case of the test samples, dilutions ranging from 0.01 through 100 μ g/mL were prepared in 0.5% DMSO.

All human tumor cell lines were obtained from the American Type Culture Collection. Cells were grown as mono layers in alpha-MEM containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin and 240 units/mL nystatin. The cells were maintained in a humidified, 5% CO₂ atmosphere at 37°C.

After trypsinization, the cells are counted and adjusted to a concentration of 50 x 10³ cells/mL (varied according to cancer cell line). 200 μ L of the cell suspension was plated into wells of 4 rows of a 96-well microtiter plate. After the cells were allowed to attach for four hours, 2 μ L of DMSO containing test sample solutions were added to quadruplicate wells. Initial dose-response finding experiments, using order of magnitude test sample dilutions were used to determine the range of doses to be examined. Well absorbancies at 540nm were then measured on

a BIO RAD MP450 plate reader. The mean absorbance of quadruplicate test sample treated wells was compared to the control, and the results expressed as the percentage of control absorbance plus/minus the standard deviation. The 5 reduction of MTT to a purple formazan product correlates in a linear manner with the number of living cells in the well. Thus, by measuring the absorbance of the reduction product, a quantitation of the percent of cell survival at a given dose of test sample can be obtained. Control wells 10 contained a final concentration of 1% DMSO.

Two of the samples were first tested by this protocol. Sample MM1 represented a very crude isolate of cocoa procyanidins and contained appreciable quantities of caffeine and theobromine. Sample MM2 represented a cocoa 15 procyanidin isolate partially purified by gel permeation chromatography. Caffeine and theobromine were absent in MM2. Both samples were screened for activity against the following cancer cell lines using the procedures previously described:

- 20 HCT 116 colon cancer
- ACHN renal adenocarcinoma
- SK-5 melanoma
- A498 renal adenocarcinoma
- MCF-7 breast cancer
- 25 PC-3 prostate cancer
- CAPAN-2 pancreatic cancer

Little or no activity was observed with MM1 on any of the cancer cell lines investigated. MM2 was found to have activity against HCT-116, PC-3 and ACHN cancer cell 30 lines. However, both MM1 and MM2 were found to interfere with MTT such that it obscured the decrease in absorbance that would have reflected a decrease in viable cell number.

This interference also contributed to large error bars, because the chemical reaction appeared to go more quickly in the wells along the perimeter of the plate. A typical example of these effects is shown in Figure 5. At the high 5 concentrations of test material, one would have expected to observe a large decrease in survivors rather than the high survivor levels shown. Nevertheless, microscopic examinations revealed that cytotoxic effects occurred, despite the MTT interference effects. For instance, an IC_{50} 10 value of $0.5\mu\text{g}/\text{mL}$ for the effect of MM2 on the ACHN cell line was obtained in this manner.

These preliminary results, in the inventors' view, required amendment of the assay procedures to preclude the interference with MTT. This was accomplished as follows. 15 After incubation of the plates at 37°C in a humidified, 5% CO_2 atmosphere for 18 hours, the medium was carefully aspirated and replaced with fresh alpha-MEM media. This media was again aspirated from the wells on the third day of the assay and replaced with $100\mu\text{L}$ of freshly prepared 20 McCoy's medium. $11\mu\text{L}$ of a 5mg/mL stock solution of MTT in PBS (Phosphate Buffered Saline) were then added to the wells of each plate. After incubation for 4 hours in a humidified, 5% CO_2 atmosphere at 37°C , $100\mu\text{L}$ of 0.04 N HCl 25 in isopropanol was added to all wells of the plate, followed by thorough mixing to solubilize the formazan produced by any viable cells. Additionally, it was decided to subfractionate the procyanidins to determine the specific components responsible for activity.

The subfractionation procedures previously 30 described were used to prepare samples for further screening. Five fractions representing the areas shown in Figure 1 and component(s) distribution shown in Figures 4A -

4E were prepared. The samples were coded MM2A through MM2E to reflect these analytical characterizations and to designate the absence of caffeine and theobromine.

Each fraction was individually screened against
5 the HCT-116, PC-3 and ACHN cancer cell lines. The results indicated that the activity did not concentrate to any one specific fraction. This type of result was not considered unusual, since the components in "active" natural product isolates can behave synergistically. In the case of the
10 cocoa procyanidin isolate (MM2), over twenty detectable components comprised the isolate. It was considered possible that the activity was related to a combination of components present in the different fractions, rather than the activity being related to an individual component(s).

15 On the basis of these results, it was decided to combine the fractions and repeat the assays against the same cancer cell lines. Several fraction combinations produced cytotoxic effects against the PC-3 cancer cell lines. Specifically, IC₅₀ values of 40µg/mL each for MM2A and MM2E
20 combination, and of 20µg/mL each for MM2C and MM2E combination, were obtained. Activity was also reported against the HCT-116 and ACHN cell lines, but as before, interference with the MTT indicator precluded precise observations. Replicate experiments were repeatedly
25 performed on the HCT-116 and ACHN lines to improve the data. However, these results were inconclusive due to bacterial contamination and exhaustion of the test sample material. Figures 6A-6D show the dose-response relationship between combinations of the cocoa extracts and PC-3 cancer cells.

30 Nonetheless, from this data, it is clear that cocoa extracts, especially cocoa polyphenols or procyanidins, have significant anti-tumor; anti-cancer or

antineoplastic activity, especially with respect to human PC-3 (prostate), HCT-116 (colon) and ACHN (renal) cancer cell lines. In addition, those results suggest that specific procyanidin fractions may be responsible for the 5 activity against the PC-3 cell line.

Example 7: Anti-Cancer, Anti-Tumor or Antineoplastic Activity of Cocoa Extracts (Procyanidins)

To confirm the above findings and further study 10 fraction combinations, another comprehensive screening was performed.

All prepared materials and procedures were identical to those reported above, except that the standard 4-replicates per test dose was increased to 8 or 12- 15 replicates per test dose. For this study, individual and combinations of five cocoa procyanidin fractions were screened against the following cancer cell lines.

PC-3 Prostate
KB Nasopharyngeal/HeLa
20 HCT-116 Colon
ACHN Renal
MCF-7 Breast
SK-5 Melanoma
A-549 Lung
25 CCRF-CEM T-cell leukemia

Individual screenings consisted of assaying different dose levels (0.01-100 μ g/mL) of fractions A, B, C, D, and E (See Figs. 4A-4E and discussion thereof, supra) against each cell line. Combination screenings consisted of 30 combining equal dose levels of fractions A+B, A+C, A+D, A+E, B+C, B+D, B+E, C+D, C+E, and D+E against each cell line. The results from these assays are individually discussed, followed by an overall summary.

5 A. PC-3 Prostate Cell Line

Figures 7A - 7H show the typical dose response relationship between cocoa procyanidin fractions and the PC-3 cell line. Figures 7D and 7E demonstrate that fractions D and E were active at an IC₅₀ value of 75µg/mL. The IC₅₀ values that were obtained from dose-response curves of the other procyanidin fraction combinations ranged between 60 - 80µg/mL when fractions D or E were present. The individual IC₅₀ values are listed in 10 Table 6.

15 B. KB Nasopharyngeal/HeLa Cell Line

Figures 8A - 8H show the typical dose response relationship between cocoa procyanidin fractions and the KB Nasopharyngeal/HeLa cell line. Figures 8D and 8E demonstrate that fractions D and E were active at an IC₅₀ value of 75µg/mL. Figures 8F - 8H depict representative results obtained from the fraction combination study. In this case, procyanidin fraction combination A+B had no effect, whereas fraction combinations B+E and D+E were active at an IC₅₀ value of 60µg/mL. The IC₅₀ values that were obtained from other dose response curves from other fraction combinations ranged from 60 - 80µg/mL when fractions D or E were present. The individual IC₅₀ values are listed in Table 6. These results were essentially the same as those obtained against the PC-3 cell line.

20 C. HCT-116 Colon Cell Line

Figure 9A - 9H show the typical dose response relationships between cocoa procyanidin fractions and the HCT-116 colon cell line. Figures 9D and 9E demonstrate that fraction E was active at an IC₅₀ value of approximately 400µg/mL. This value was obtained by extrapolation of the existing curve. Note that the slope of the dose response

curve for fraction D also indicated activity. However, no IC₅₀ value was determined from this plot, since the slope of the curve was too shallow to obtain a reliable value.

Figures 9F - 9H depict representative results obtained from 5 the fraction combination study. In this case, procyanidin fraction combination B+D did not show appreciable activity, whereas fraction combinations A+E and D+E were active at IC₅₀ values of 500µg/mL and 85µg/mL, respectively. The IC₅₀ values that were obtained from dose response curves of other 10 fraction combinations averaged about 250µg/mL when fraction E was present. The extrapolated IC₅₀ values are listed in Table 6.

D. ACHN Renal Cell Line

Figure 10A - 10H show the typical dose response 15 relationships between cocoa procyanidin fractions and the ACHN renal cell line. Figures 10A - 10E indicated that no individual fraction was active against this cell line.

Figures 10F - 10H depict representative results obtained from the fraction combination study. In this case, 20 procyanidin fraction combination B+C was inactive, whereas the fraction combination A+E resulted in an extrapolated IC₅₀ value of approximately 500µg/mL. Dose response curves similar to the C+D combination were considered inactive, since their slopes were too shallow. Extrapolated IC₅₀ 25 values for other fraction combinations are listed in Table 6.

E. A-549 Lung Cell Line

Figures 11A - 11H show the typical dose response 30 relationships between cocoa procyanidin fractions and the A-549 lung cell line. No activity could be detected from any individual fraction or combination of fractions at the doses

used in the assay. However, procyanidin fractions may nonetheless have utility with respect to this cell line.

F. SK-5 Melanoma Cell Line

Figure 12A - 12H show the typical dose response relationships between cocoa procyanidin fractions and the SK-5 melanoma cell line. No activity could be detected from any individual fraction or combination of fractions at the doses used in the assay. However, procyanidin fractions may nonetheless have utility with respect to this cell line.

G. MCF-7 Breast Cell Line

Figures 13A - 13H show the typical dose response relationships between cocoa procyanidin fractions and the MCF-7 breast cell line. No activity could be detected from any individual fraction or combination of fractions at the doses used in the assay. However, procyanidin fractions may nonetheless have utility with respect to this cell line.

H. CCRF-CEM T-Cell Leukemia Line

A typical dose response curves were originally obtained against the CCRF-CEM T-cell leukemia line. However, microscopic counts of cell number versus time at different fraction concentrations indicated that 500 µg of fractions A, B and D effected an 80% growth reduction over a four day period. A representative dose response relationship is shown in Figure 14.

I. Summary

The IC₅₀ values obtained from these assays are collectively listed in Table 6 for all the cell lines except for CCRF-CEM T-cell leukemia. The T-cell leukemia data was intentionally omitted from the Table, since a different assay procedure was used. A general summary of these results indicated that the most activity was associated with fractions D and E. These fractions were most active against

the PC-3 (prostate) and KB (nasopharyngeal/HeLa) cell lines. These fractions also evidenced activity against the HCT-116 (colon) and ACHN (renal) cell lines, albeit but only at much higher doses. No activity was detected against the MCF-7 (breast), SK-5 (melanoma) and A-549 (lung) cell lines.

5 However, procyanidin fractions may nonetheless have utility with respect to these cell lines. Activity was also shown against the CCRF-CEM (T-cell leukemia) cell line. It should also be noted that fractions D and E are the most complex

10 compositionally. Nonetheless, from this data it is clear that cocoa extracts, especially cocoa procyanidins, have significant anti-tumor, anti-cancer or antineoplastic activity.

Table 6: IC₅₀ Values for Cocoa Procyanidin Fractions
Against Various Cell Lines

(IC₅₀ values in µg/mL)

5

10

15

20

FRACTION	PC-3	KB	HCT-116	ACHN	MCF-7	SK-5	A-549
A							
B							
C							
D	90	80					
E	75	75	400				
A+B							
A+C	125	100					
A+D	75	75					
A+E	80	75	500	500			
B+C							
B+D	75	80					
B+E	60	65	200				
C+D	80	75		1000			
C+E	80	70	250				
D+E	80	60	85				

Values above 100µg/mL were extrapolated from
dose response curves

25 Example 8. Anti-Cancer, Anti-Tumor or Antineoplastic
Activity of Cocoa Extracts (Procyanidins)

Several additional *in vitro* assay procedures were
used to complement and extend the results presented in

30 Examples 6 and 7.

Method A. Crystal Violet Staining Assay

All human tumor cell lines were obtained from the American Type Culture Collection. Cells were grown as monolayers in IMEM containing 10% fetal bovine serum without 5 antibiotics. The cells were maintained in a humidified, 5% CO₂ atmosphere at 37°C.

After trypsinization, the cells were counted and adjusted to a concentration of 1,000-2,000 cells per 100 mL. Cell proliferation was determined by plating the cells 10 (1,000-2,000 cells/well) in a 96 well microtiter plate. After addition of 100 μ L cells per well, the cells were allowed to attach for 24 hours. At the end of the 24 hour period, various cocoa fractions were added at different concentrations to obtain dose response results. The cocoa 15 fractions were dissolved in media at a 2 fold concentration and 100 μ L of each solution was added in triplicate wells. On consecutive days, the plates were stained with 50 μ L crystal violet (2.5g crystal violet dissolved in 125mL methanol, 375mL water), for 15 min. The stain was removed 20 and the plate was gently immersed into cold water to remove excess stain. The washings were repeated two more times, and the plates allowed to dry. The remaining stain was solubilized by adding 100 μ L of 0.1M sodium citrate/50% ethanol to each well. 25 After solubilization, the number of cells were quantitated on an ELISA plate reader at 540nm (reference filter at 410nm). The results from the ELISA reader were graphed with absorbance on the y-axis and days growth on the x-axis.

Method B. Soft Agar Cloning Assay

30 Cells were cloned in soft agar according to the method described by Nawata et al. (1981). Single cell suspensions were made in media containing 0.8% agar with

various concentrations of cocoa fractions. The suspensions were aliquoted into 35mm dishes coated with media containing 1.0% agar. After 10 days incubation, the number of colonies greater than 60 μ m in diameter were determined on an
5 Ominicron 3600 Image Analysis System. The results were plotted with number of colonies on the y-axis and the concentrations of a cocoa fraction on the x-axis.

Method C. XTT-Microculture Tetrazolium Assay

The XTT assay procedure described by Scudiero et al. (1988) was used to screen various cocoa fractions. The XTT assay was essentially the same as that described using the MTT procedure (Example 6) except for the following modifications. XTT ((2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-((phenylamino)carbonyl)-2H-tetrazolium
10 hydroxide) was prepared at 1mg/mL medium without serum, prewarmed to 37°C. PMS was prepared at 5mM PBS. XTT and PMS were mixed together; 10 μ L of PMS per mL XTT and 50 μ L PMS-XTT were added to each well. After an incubation at
15 37°C for 4 hr, the plates were mixed 30 min. on a mechanical shaker and the absorbance measured at 450-600nm. The results were plotted with the absorbance on the y-axis and days growth or concentration on the x-axis.
20
25

For methods A and C, the results were also plotted as the percent control as the y-axis and days growth or concentration on the x-axis.

A comparison of the XTT and Crystal Violet Assay procedures was made with cocoa fraction D & E (Example 3B) against the breast cancer cell line MCF-7 p168 to determine which assay was most sensitive. As shown in Figure 15A,
30 both assays showed the same dose-response effects for concentrations >75 μ g/mL. At concentrations below this value, the crystal violet assay showed higher standard

deviations than the XTT assay results. However, since the crystal violet assay was easier to use, all subsequent assays, unless otherwise specified, were performed by this procedure.

- 5 Crystal violet assay results are presented (Figures 15B-15E) to demonstrate the effect of a crude polyphenol extract (Example 2) on the breast cancer cell line MDA MB231, prostate cancer cell line PC-3, breast cancer cell line MCF-7 p163, and cervical cancer cell line
10 Hela, respectively. In all cases a dose of 250 μ g/mL completely inhibited all cancer cell growth over a period of 5-7 days. The Hela cell line appeared to be more sensitive to the extract, since a 100 μ g/mL dose also inhibited growth. Cocoa fractions from Example 3B were also assayed against
15 Hela and another breast cancer cell line SKBR-3. The results (Figures 15F and 15G) showed that fraction D & E has the highest activity. As shown in Figures 15H and 15I, IC₅₀ values of about 40 μ g/mL D & E were obtained from both cancer cell lines.
20 The cocoa fraction D & E was also tested in the soft agar cloning assay which determines the ability of a test compound(s) to inhibit anchorage independent growth. As shown in Figure 15J, a concentration of 100 μ g/mL completely inhibited colony formation of Hela cells.
25 Crude polyphenol extracts obtained from eight different cocoa genotypes representing the three horticultural races of cocoa were also assayed against the Hela cell line. As shown in Figure 15K all cocoa varieties showed similar dose-response effects. The
30 UIT-1 variety exhibited the most activity against the Hela cell line. These results demonstrated that all cocoa genotypes possess a polyphenol fraction that elicits

activity against at least one human cancer cell line that is independent of geographical origin, horticultural race, and genotype.

Another series of assays were performed on crude polyphenol extracts prepared on a daily basis from a one ton scale traditional 5-day fermentation of Brazilian cocoa beans, followed by a 4-day sun drying stage. The results shown in Figure 15L showed no obvious effect of these early processing stages, suggesting little change in the composition of the polyphenols. However, it is known (Lehrian and Patterson, 1983) that polyphenol oxidase (PPO) will oxidize polyphenols during the fermentation stage. To determine what effect enzymatically oxidized polyphenols would have on activity, another experiment was performed.

Crude PPO was prepared by extracting finely ground, unfermented, freeze dried, defatted Brazilian cocoa beans with acetone at a ratio of 1gm powder to 10mL acetone. The slurry was centrifuged at 3,000 rpm for 15 min. This was repeated three times, discarding the supernatant each time with the fourth extraction being poured through a Buchner filtering funnel. The acetone powder was allowed to air dry, followed by assay according to the procedures described by McLord and Kilara, (1983). To a solution of crude polyphenols (100mg/10mL Citrate-Phosphate buffer, 0.02M, pH 5.5) 100mg of acetone powder (4,000 units activity/mg protein) was added and allowed to stir for 30 min. with a stream of air bubbled through the slurry. The sample was centrifuged at 5,000xg for 15 min. and the supernatant extracted 3X with 20mL ethyl acetate. The ethyl acetate extracts were combined, taken to dryness by distillation under partial vacuum and 5mL water added, followed by lyophilization. The material was then assayed against Hela

cells and the dose-response compared to crude polyphenol extracts that were not enzymatically treated. The results (Figure 15M) showed a significant shift in the dose-response curve for the enzymatically oxidized extract, showing that 5 the oxidized products were more inhibitory than their native forms.

**Example 9: Antioxidant Activity of Cocoa Extracts
 Containing Procyanidins**

10 Evidence in the literature suggests a relationship between the consumption of naturally occurring antioxidants (Vitamins C, E and Beta-carotene) and a lowered incidence of disease, including cancer (Designing Foods, 1993; Caragay, 1992). It is generally thought that these antioxidants 15 affect certain oxidative and free radical processes involved with some types of tumor promotion. Additionally, some plant polyphenolic compounds that have been shown to be anticarcinogenic, also possess substantial antioxidant activity (Ho et al., 1992; Huang et al., 1992).

20 To determine whether cocoa extracts containing procyanidins possessed antioxidant properties, a standard Rancimat method was employed. The procedures described in Examples 1, 2 and 3 were used to prepare cocoa extracts which were manipulated further to produce two fractions from 25 gel permeation chromatography. These two fractions are actually combined fractions A through C, and D and E (See Figure 1) whose antioxidant properties were compared against the synthetic antioxidants BHA and BHT.

20 Peanut Oil was pressed from unroasted peanuts after the skins were removed. Each test compound was spiked into the oil at two levels, ~ 100 ppm and ~ 20 ppm, with the actual levels given in Table 7. 50 μ L of methanol solubilized antioxidant was added to each sample to aid in

dispersion of the antioxidant. A control sample was prepared with 50 μ L of methanol containing no antioxidant.

The samples were evaluated in duplicate, for oxidative stability using the Rancimat stability test at 5 100°C and 20 cc/min of air. Experimental parameters were chosen to match those used with the Active Oxygen Method (AOM) or Swift Stability Test (Van Oosten et al., 1981). A typical Rancimat trace is shown in Figure 16. Results are reported in Table 8 as hours required to reach a peroxide level of 100 meq.

10

Table 7: Concentrations of Antioxidants

	SAMPLE	LEVEL 1	LEVEL 2
		ppm	
15	Butylated Hydroxytoluene (BHT)	24	120
	Butylated Hydroxyanisole (BHA)	24	120
20	Crude Ethyl Acetate Fraction of Cocoa	22	110
	Fraction A-C	20	100
	Fraction D-E	20	100

Table 8: Oxidative Stability of Peanut Oil with Various Antioxidants

	SAMPLE	20 ppm	100 ppm
		average	
	Control		10.5 \pm 0.7
	BHT	16.5 \pm 2.1	12.5 \pm 2.1
30	BHA	13.5 \pm 2.1	14.0 \pm 1.4
	Crude Cocoa Fraction	18.0 \pm 0.0	19.0 \pm 1.4

Fraction A-C	16.0 ± 6.4	17.5 ± 0.0
Fraction D-E	14.0 ± 1.4	12.5 ± 0.7

These results demonstrated increased oxidative stability of peanut oil with all of the additives tested. The highest increase in oxidative stability was realized by the sample spiked with the crude ethyl acetate extract of cocoa. These results demonstrated that cocoa extracts containing procyanidins have antioxidant potential equal to or greater than equal amounts of synthetic BHA and BHT. Accordingly, the invention may be employed in place of BHT or BHA in known utilities of BHA or BHT, for instance as an antioxidant and/or food additive. And, in this regard, it is noted too that the invention is from an edible source. Given these results, the skilled artisan can also readily determine a suitable amount of the invention to employ in such "BHA or BHT" utilities, e.g., the quantity to add to food, without undue experimentation.

Example 10: Topoisomerase II Inhibition Study

DNA topoisomerase I and II are enzymes that catalyze the breaking and rejoining of DNA strands, thereby controlling the topological states of DNA (Wang, 1985). In addition to the study of the intracellular function of topoisomerase, one of the most significant findings has been the identification of topoisomerase II as the primary cellular target for a number of clinically important antitumor compounds (Yamashita et al., 1990) which include intercalating agents (m-AMSA, Adriamycin® and ellipticine) as well as nonintercalating epipodophyllotoxins. Several lines of evidence indicate that some antitumor drugs have the common property of stabilizing the DNA - topoisomerase II complex ("cleavable complex") which upon exposure to

denaturing agents results in the induction of DNA cleavage (Muller et al., 1989). It has been suggested that the cleavable complex formation by antitumor drugs produces bulky DNA adducts that can lead to cell death.

5 According to this attractive model, a specific new inducer of DNA topoisomerase II cleavable complex is useful as an anti-cancer, anti-tumor or antineoplastic agent. In an attempt to identify cytotoxic compounds with activities that target DNA, the cocoa procyanidins were screened for
10 enhanced cytotoxic activity against several DNA - damage sensitive cell lines and enzyme assay with human topoisomerase II obtained from lymphoma.

A. Decatenation of Kinetoplast DNA by Topoisomerase II

15 The *in vitro* inhibition of topoisomerase II decatenation of kinetoplast DNA, as described by Muller et al. (1989), was performed as follows. Nuclear extracts containing topoisomerase II activity were prepared from human lymphoma by modifications of the methods of Miller et
20 al. (1981) and Danks et al. (1988). One unit of purified enzyme was enough to decatenate 0.25 µg of kinetoplast DNA in 30 min. at 34°C. Kinetoplast DNA was obtained from the trypanosome *Crithidia fasciculata*. Each reaction was carried out in a 0.5mL microcentrifuge tube containing
25 19.5µL H₂O, 2.5µL 10X buffer (1X buffer contains 50mM tris-HCl, pH 8.0, 120mM KCl, 10mM MgCl₂, 0.5mM ATP, 0.5mM dithiothreitol and 30µg BSA/mL), 1µL kinetoplast DNA (0.2µg), and 1µL DMSO-containing cocoa procyanidin test fractions at various concentrations. This combination was
30 mixed thoroughly and kept on ice. One unit of topoisomerase was added immediately before incubation in a waterbath at 34°C for 30 min.

Following incubation, the decatenation assay was stopped by the addition of 5 μ L stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol) and placed on ice. DNA was electrophoresed on a 1% agarose gel in TAE buffer containing ethidium bromide (0.5 μ g/mL). Ultraviolet illumination at 310nm wavelength allowed the visualization of DNA. The gels were photographed using a Polaroid Land camera.

- 5 Figure 17 shows the results of these experiments.
- 10 Fully catenated kinetoplast DNA does not migrate into a 1% agarose gel. Decatenation of kinetoplast DNA by topoisomerase II generates bands of monomeric DNA (monomer circle, forms I and II) which do migrate into the gel. Inhibition of the enzyme by addition of cocoa procyanidins
- 15 is apparent by the progressive disappearance of the monomer bands as a function of increasing concentration. Based on these results, cocoa procyanidin fractions A, B, D, and E were shown to inhibit topoisomerase II at concentrations ranging from 0.5 to 5.0 μ g/mL. These inhibitor
- 20 concentrations were very similar to those obtained for mitoxanthrone and m-AMSA (4'-(9-acridinylamino)methanesulfon-m-anisidine).

B. Drug Sensitive Cell Lines

Cocoa procyanidins were screened for cytotoxicity

25 against several DNA-damage sensitive cell lines. One of the cell lines was the xrs-6 DNA double strand break repair mutant developed by P. Jeggo (Kemp et al., 1984). The DNA repair deficiency of the xrs-6 cell line renders them particularly sensitive to x-irradiation, to compounds that

30 produce DNA double strand breaks directly, such as bleomycin, and to compounds that inhibit topoisomerase II, and thus may indirectly induce double strand breaks as

suggested by Warters et al. (1991). The cytotoxicity toward the repair deficient line was compared to the cytotoxicity against a DNA repair proficient CHO line, BR1. Enhanced cytotoxicity towards the repair deficient (xrs-6) line was
5 interpreted as evidence for DNA cleavable double strand break formation.

The DNA repair competent CHO line, BR1, was developed by Barrows et al. (1987) and expresses O⁶-alkylguanine - DNA - alkyltransferase in addition to normal
10 CHO DNA repair enzymes. The CHO double strand break repair deficient line (xrs-6) was a generous gift from Dr. P. Jeggo and co-workers (Jeggo et al., 1989). Both of these lines were grown as monolayers in alpha-MEM containing serum and antibiotics as described in
15 Example 6. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. Before treatment with cocoa procyanidins, cells grown as monolayers were detached with trypsin treatment. Assays were performed using the MTT assay procedure described in Example 6.

20 The results (Figure 18) indicated no enhanced cytotoxicity towards the xrs-6 cells suggesting that the cocoa procyanidins inhibited topoisomerase II in a manner different from cleavable double strand break formation. That is, the cocoa procyanidins interact with topoisomerase
25 II before it has interacted with the DNA to form a noncleavable complex.

Noncleavable complex forming compounds are relatively new discoveries. Members of the anthracyclines, podophyllin alkaloids, anthracenediones, acridines, and
30 ellipticines are all approved for clinical anti-cancer, anti-tumor or antineoplastic use, and they produce cleavable complexes (Liu, 1989). Several new classes of topoisomerase

II inhibitors have recently been identified which do not appear to produce cleavable complexes. These include amonafide (Hsiang et al., 1989), distamycin (Fesen et al., 1989), flavanoids (Yamashita et al., 1990), saintopin (Yamashita et al., 1991), membranone (Drake et al., 1989), terpenoids (Kawada et al., 1991), anthrapyrazoles (Fry et al., 1985), dioxopiperazines (Tanabe et al., 1991), and the marine acridine - dercitin (Burres et al., 1989).

Since the cocoa procyanidins inactivate topoisomerase II before cleavable complexes are formed, they have chemotherapy value either alone or in combination with other known and mechanistically defined topoisomerase II inhibitors. Additionally, cocoa procyanidins also appear to be a novel class of topoisomerase II inhibitors, (Kashiwada et al., 1993) and may thus be less toxic to cells than other known inhibitors, thereby enhancing their utility in chemotherapy.

The human breast cancer cell line MCF-7 (ADR) which expresses a membrane bound glycoprotein (gp170) to confer multi-drug resistance (Leonessa et al., 1994) and its parental line MCF-7 p168 were used to assay the effects of cocoa fraction D & E. As shown in Figure 19, the parental line was inhibited at increasing dose levels of fraction D & E, whereas the Adriamycin (ADR) resistant line was less effected at the higher doses. These results show that cocoa fraction D & E has an effect on multi-drug resistant cell lines.

Example 11: Synthesis of Procyanidins

The synthesis of procyanidins was performed according to the procedures developed by Delcour et al. (1983), with modification. In addition to condensing

(+)-catechin with dihydroquercetin under reducing conditions, (-)-epicatechin was also used to reflect the high concentrations of (-)-epicatechin that naturally occur in unfermented cocoa beans. The synthesis products were 5 isolated, purified, analyzed, and identified by the procedures described in Examples 3, 4 and 5. In this manner, the biflavonoids, triflavonoids and tetraflavonoids are prepared and used as analytical standards and, in the manner described above with respect to cocoa extracts.

10 Example 12: Assay of Normal Phase Semi-Preparative Fractions

Since the polyphenol extracts are compositionally complex, it was necessary to determine which components were 15 active against cancer cell lines for further purification, dose-response assays and comprehensive structural identification. A normal phase semi preparative HPLC separation (Example 3B) was used to separate cocoa procyanidins on the basis of oligomeric size. In addition 20 to the original extract, twelve fractions were prepared (Figures 2B and 15 O) and assayed at 100 μ g/mL and 25 μ g/mL doses against HeLa and SKBR-3 cancer cell lines to determine which oligomer possessed the greatest activity. As shown in Figures 20A and B, fractions 4-11 (pentamer-dodecamer) 25 significantly inhibited HeLa and SKBr-3 cancer cell lines at the 100 μ g/mL level. These results indicated that these specific oligomers had the greatest activity against HeLa and SKBR-3 cells. Additionally, normal phase HPLC analysis of cocoa fraction D & E indicated that this fraction, used 30 in previous investigations, e.g., Example 7, was enriched with these oligomers.

Example 13: HPLC Purification Methods
Method A. GPC Purification

Procyanidins obtained as in Example 2 were partially purified by liquid chromatography on Sephadex LH 5 20 (72.5 x 2.5cm), using 100% methanol as the eluting solvent, at a flow rate of 3.5mL/min. Fractions of the eluent were collected after the first 1.5 hours, and the fractions were concentrated by a rotary evaporator, redissolved in water and freeze dried. These fractions were 10 referred to as pentamer enriched fractions. Approximately 2.00g of the extract obtained from Example 2 was subfractionated in this manner. Results are shown in Table 9.

Table 9: Composition of Fractions Obtained:

Fraction (Time)	Monomer (% Area)	Dimer (% Area)	Trimer (% Area)	Tetramer (% Area)	Pentamer (% Area)	Hexamer (% Area)	Heptamer (% Area)	Octamer (% Area)	Nonamer (% Area)	Decamer (% Area)	Undecamer (% Area)	Others (% Area)
1:15	73	8	16	3	ND	ND	ND	ND	ND	ND	ND	ND
1:44	67	19	10	3	1	tr	tr	tr	tr	tr	tr	tr
2:13	30	29	24	11	4	1	tr	tr	tr	tr	tr	tr
2:42	2	16	31	28	15	6	2	tr	tr	tr	tr	tr
3:11	1	12	17	25	22	13	7	2	1	tr	tr	tr
3:40	tr	18	13	18	20	15	10	5	2	tr	tr	tr
4:09	tr	6	8	17	21	19	14	8	4	2	tr	tr

ND = not detected
 tr = trace amount

Method B. Normal Phase Separation

Procyanidins obtained as Example 2 were separated purified by normal phase chromatography on Supelcosil LC-Si, 100Å, 5µm (250 x 4.6mm), at a flow rate of 1.0mL/min, or, in
5 the alternative, Lichrosphere® Silica 100, 100Å, 5µm (235 x 3.2mm), at a flow rate of 0.5mL/min. Separations were aided by a step gradient under the following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86). Mobile phase composition was A =
10 dichloromethane; B = methanol; and C = acetic acid:water (1:1). Components were detected by fluorescence where $\lambda_{ex} = 276\text{nm}$ and $\lambda_{em} = 316\text{nm}$, and by UV at 280nm. The injection volume was 5.0µL (20mg/mL) of the procyanidins obtained from Example 2. These results are shown in Fig.
15 40A and 40B.

In the alternative, separations were aided by a step gradient under the following conditions: (Time, %A, %B); (0, 76, 20); (25, 46, 50); (30, 10, 86). Mobile phase composition was A = dichloromethane; B = methanol; and C = acetic acid : water (1:1). The results are shown in Fig.
20 41A and 41B.

Method C. Reverse - Phase Separation

Procyanidins obtained as in Example 2 were separated purified by reverse phase chromatography on
25 Hewlett Packard Hypersil ODS 5µm. (200 x 2.1mm), and a Hewlett Packard Hypersil ODS 5µm guard column (20 x 2.1mm). The procyanidins were eluted with a linear gradient of 20% B into A in 20 minutes, followed by a column wash with 100% B at a flow rate of 0.3mL/min. The mobile phase composition
30 was a degassed mixture of B = 1.0% acetic acid in methanol and A = 2.0% acetic acid in nanopure water. Components were detected by UV at 280nm, and fluorescence where $\lambda_{ex} = 276\text{nm}$

and $\lambda_{\text{em}} = 316\text{nm}$; and the injection volume was $2.0\mu\text{l}$ (20mg/mL).

Example 14: HPLC Separation of Pentamer Enriched Fractions

5

Method A. Semi-Preparative Normal Phase HPLC

The pentamer enriched fractions were further purified by semi-preparative normal phase HPLC by a Hewlett Packard 1050 HPLC system equipped with a Millipore - Waters model 480 LC detector set at 254nm, which was assembled with a Pharmacia Frac-100 Fraction Collector set to peak mode. Separations were effected on a Supelco $5\mu\text{m}$ Supelcosel LC-Si, 100\AA column ($250 \times 10\text{mm}$) connected with a Supelco 5μ Supelguard LC-Si guard column ($20 \times 4.6\text{mm}$). Procyanidins were eluted by a linear gradient under the following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86) followed by a 10 minute re-equilibration. Mobile phase composition was A = dichloromethane; B = methanol; and C = acetic acid:water (1:1). A flow rate of 3mL/min was used. Components were detected by UV at 254nm; and recorded on a Kipp & Zonan BD41 recorder. Injection volumes ranged from $100\text{-}250\mu\text{l}$ of 10mg of procyanidin extracts dissolved in 0.25mL 70% aqueous acetone. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further purification and subsequent evaluation.

HPLC conditions: 250 x 100mm Supelco Supelcosil LC-Si
 (5 μ m) Semipreparative Column
 20 x 4.6mm Supelco Supelcosil LC-Si
 (5 μ m) Guard Column

5

Detector: Waters LC
 Spectrophotometer Model
 480 @ 254nm

Flow rate: 3mL/min.,
 10 Column Temperature: ambient,
 Injection: 250 μ l of pentamer enriched
 extract

	Gradient:	CH ₂ Cl ₂	methanol	acetic acid: water (1:1)
15	0	82	14	4
	30	67.6	28.4	4
	60	46	50	4
	65	10	86	4
20	70	10	86	4

Method B. Reverse Phase Separation

Procyanidin extracts obtained as in Example 13
 25 were filtered through a 0.45 μ nylon filter and analyzed by a
 Hewlett Packard 1090 ternary phase HPLC system equipped with
 a Diode Array detector and a HP model 1046A Programmable
 Fluorescence Detector. Separations were effected at 45°C on
 a Hewlett Packard 5 μ Hypersil ODS column (200 x 2.1mm). The
 30 procyanidins were eluted with a linear gradient of 60% B
 into A followed by a column wash with B at a flow rate of
 0.3mL/min. The mobile phase composition was a de-gassed
 mixture of B = 0.5% acetic acid in methanol and A = 0.5%
 acetic acid in nanopure water. Acetic acid levels in A and
 35 B mobile phases can be increased to 2%. Components were
 detected by fluorescence, where λ_{ex} = 276nm and λ_{em} = 316nm,
 and by UV at 280nm. Concentrations of (+)-catechin and (-)-
 epicatechin were determined relative to reference standard

solutions. Procyanidin levels were estimated by using the response factor for (-)-epicatechin.

Method C. Normal Phase Separation

Pentamer enriched procyanidin extracts obtained as
5 in Example 13 were filtered through a 0.45μ nylon filter and analyzed by a Hewlett Packard 1090 Series II HPLC system equipped with a HP Model 1046A Programmable Fluorescence detector and Diode Array detector. Separations were effected at 37°C on a 5μ Phenomenex Lichrosphere[®] Silica 100
10 column ($250 \times 3.2\text{mm}$) connected to a Supelco Supelguard LC-Si 5μ guard column ($20 \times 4.6\text{mm}$). Procyanidins were eluted by linear gradient under the following conditions: (time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86), followed by an 8 minute re-equilibration.
15 Mobile phase composition was A = dichloromethane, B = methanol, and C = acetic acid:water at a volume ratio of 1:1. A flow rate of 0.5mL/min was used. Components were detected by fluorescence, where $\lambda_{\text{ex}} = 276\text{nm}$ and $\lambda_{\text{em}} = 316\text{nm}$ or by UV at 280nm . A representative HPLC chromatogram
20 showing the separation of the various procyanidins is shown in Figure 2 for one genotype. Similar HPLC profiles were obtained from other *Theobroma*, *Herrania* and/or their inter or intra specific crosses.

HPLC conditions:

25 $250 \times 3.2\text{mm}$ Phenomenex Lichrosphere[®] Silica 100 column (5μ) $20 \times 4.6\text{mm}$ Supelco Supelguard LC-Si (5μ) guard column
30 Detectors: Photodiode Array @ 280nm
 Fluorescence $\lambda_{\text{ex}} = 276\text{nm}$; $\lambda_{\text{em}} = 316\text{nm}$
 Flow rate: 0.5 mL/min .
 Column temperature: 37°C

Gradient:	CH ₂ Cl ₂	methanol	acetic acid: water (1:1)
5	0	82	14
	30	67.6	28.4
	60	46	50
	65	10	86
	70	10	86

Method D. Preparative Normal Phase Separation

10 The pentamer enriched fractions obtained as in Example 13 were further purified by preparative normal phase chromatography by modifying the method of Rigaud et al., (1993) J. Chrom. 654, 255-260.

15 Separations were affected at ambient temperature on a 5μ Supelcosil LC-Si 100Å column (50 x 2cm), with an appropriate guard column. Procyanidins were eluted by a linear gradient under the following conditions: (time, %A, %B, flow rate); (0, 92.5, 7.5, 10); (10, 92.5, 7.5, 40); (30, 91.5, 18.5, 40); (145, 88, 22, 40); (150, 24, 86, 40); (20, 155, 24, 86, 50); (180, 0, 100, 50). Prior to use, the mobile phase components were mixed by the following protocol:

Solvent A preparation (82% CH₂Cl₂, 14% methanol, 2% acetic acid, 2% water):

25 1. Measure 80mL of water and dispense into a 4L bottle.

2. Measure 80mL of acetic acid and dispense into the same 4L bottle.

3. Measure 560mL of methanol and dispense into the same 4L bottle.

30 4. Measure 3280mL of methylene chloride and dispense into the 4L bottle.

5. Cap the bottle and mix well.

6. Purge the mixture with high purity Helium for 5-10 minutes to degas.

Repeat steps 1-6 two times to yield 8 volumes of solvent A.

Solvent B preparation (96% methanol, 2% acetic acid, 2% water):

- 5 1. Measure 80mL of water and dispense into a 4L bottle.
2. Measure 80mL of acetic acid and dispense into the same 4L bottle.
3. Measure 3840mL of methanol and dispense 3840mL of methanol and dispense into the same 4L bottle.
4. Cap the bottle and mix well.
5. Purge the mixture with high purity Helium for 5-10 minutes to degas.

Repeat steps 1-5 to yield 4 volumes of solvent B.

- 15 Mobile phase composition was A = methylene chloride with 2% acetic acid and 2% water; B = methanol with 2% acetic acid and 2% water. The column load was 0.7g in 7mL. components were dected by UV at 254nm. A typical preparative normal phase HPLC separation of cocoa procyanidins is shown in
- 20 Figure 42.

HPLC Conditions:

Column: 50 x 2cm 5 μ Supelcosil LC-Si run @ ambient temperature.

- 25 Mobile Phase: A = Methylene Chloride with 2% Acetic Acid and 2% Water.

B = Methanol with 2% Acetic Acid and 2% Water.

30

Gradient/Flow Profile:

	TIME (MIN)	%A	%B	FLOW RATE (mL/min)
5	0	92.5	7.5	10
	10	92.5	7.5	40
	30	91.5	8.5	40
	145	88.0	22.0	40
	150	24.0	86.0	40
	155	24.0	86.0	50
10	180	0.0	100.0	50

Example 15: Identification of Procyanidins

15 Procyanidins obtained as in Example 14, method D were analyzed by Matrix Assisted Laser Desorption Ionization-Time of Flight/Mass Spectrometry (MALDI-TOF/MS) using a HP G2025A MALDI-TOF/MS system equipped with a Lecroy 9350 500 MHz Oscilloscope. The instrument was calibrated in accordance with the manufacturer's instructions with a low molecular weight peptide standard (HP Part No. G2051A) or peptide standard (HP Part No. G2052A) with 2,5-dihydroxybenzoic acid (DHB)(HP Part No. G2056A) as the sample matrix. One (1.0) mg of sample was dissolved in 25 500 μ l of 70/30 methanol/water, and the sample was then mixed with DHB matrix, at a ratio of 1:1, 1:10 or 1:50 (sample:matrix) and dried on a mesa under vacuum. The samples were analyzed in the positive ion mode with the detector voltage set at 4.75kV and the laser power set between 1.5 and 8 μ J. Data was collected as the sum of a number of single shots and displayed as units of molecular weight and time of flight. A representative MALDI-TOF/MS is shown in Figure 22A.

Figures 22 and C show MALDI-TOF/MS spectra obtained from partially purified procyanidins prepared as described in Example 3, Method A and used for *in vitro* assessment as described in Examples 6 and 7, and whose 5 results are summarized in Table 6. This data illustrates that the inventive compounds described herein were predominantly found in fractions D-E, but not A-C.

The spectra were obtained as follows:

The purified D-E fraction was subjected to MALDI-
10 TOF/MS as described above, with the exception that the fraction was initially purified by SEP-PACK® C-18 cartridge. Five (5) mg of fraction D-E in 1 mL nanopure water was loaded onto a pre-equilibrated SEP-PACK® cartridge. The column was washed with 5mL nanopure water to eliminate
15 contaminants, and procyanidins were eluted with 1mL 20% methanol. Fractions A-C were used directly, as they were isolated in Example 3, Method A, without further purification.

These results confirmed and extended earlier
20 results (see Example 5, Table 3, Figs. 20A and B) and indicate that the inventive compounds have utility as sequestrants of cations. In particular, MALDI-TOF/MS results conclusively indicated that procyanidin oligomers of n = 5 and higher (see Figures 20A and B; and formula under
25 Objects and Summary of the Invention) were strongly associated with anti-cancer activity with the HeLa and SKBR-3 cancer cell line model. Oligomers of n = 4 or less were ineffective with these models. The pentamer structure apparently has a structural motif which is present in it and
30 in higher oligomers which provides the activity. Additionally, it was observed that the MALDI-TOF/MS data

showed strong M⁺ ions of Na⁺, 2 Na⁺, K⁺, 2 K⁺, Ca⁺⁺, demonstrating the utility as cation sequestrants.

Example 16: Purification of Oligomeric Fractions

Method A. Purification by Semi-Preparative

5 Reverse Phase HPLC

Procyanidins obtained from Example 14, Method A and B and D were further separated to obtain experimental quantities of like oligomers for further structural identification and elucidation (e.g., Example 15, 18, 19, and 20). A Hewlett Packard 1050 HPLC system equipped with a variable wavelength detector, Rheodyne 7010 injection valve with 1mL injection loop was assembled with a Pharmacia FRAC-100 Fraction Collector. Separations were effected on a Phenomenex Ultracarb[®] 10 μ ODS column (250 x 22.5mm) connected with a Phenomenex 10 μ ODS Ultracarb[®] (60 x 10mm) guard column. The mobile phase composition was A = water; B = methanol used under the following linear gradient conditions: (time, %A); (0,85), (60,50), (90,0 and (110,0) at a flow rate of 5 mL/min. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further evaluation by MALDI-TOF/MS and NMR. Injection loads ranged from 25-100mg of material. A representative elution profile is shown in Fig. 23b.

25 Method B. Modified Semi-Preparative HPLC

Procyanidins obtained from Example 14, Method A and B and D were further separated to obtain experimental quantities of like oligomers for further structural identification and elucidation (e.g., Example 15, 18, 19, and 20). Supelcosil LC-Si 5 μ column (250 x 10mm) with a Supelcosil LC-Si 5 μ (20 x 2mm) guard column. The

separations were effected at a flow rate of 3.0mL/min, at ambient temperature. The mobile phase composition was A = dichloromethane; B = methanol; and C = acetic acid:water (1:1); used under the following linear gradient conditions:

5 (time, %A, %B); (0, 82, 14); (22, 74, 21); (32, 74, 21);
(60, 74, 50, 4); (61, 82, 14), followed by column re-equilibration for 7 minutes. Injection volumes were 60 μ l containing 12mg of enriched pentamer. Components were detected by UV at 280nm. A representative elution profile
10 is shown in Figure 23A.

Example 17: Molecular Modeling of Pentamers

Energy minimized structures were determined by molecular modeling using Desktop Molecular Modeller, version 3.0, Oxford University Press, 1994. Four representative views of (EC(4 → 8))₄EC (EC = epicatechin) pentamers based on the structure of epicatechin are shown in Figures 24 A-D. A helical structure is suggested. In general when epicatechin is the first monomer and the bonding is 4-8, a beta configuration results, when the first monomer is catechin and the bonding is 4-8, an alpha configuration results; and, these results are obtained regardless of whether the second monomer is epicatechin or catechin (an exception is ent-EC(4 → 8)ent-EC). Figures 38A - 38P show preferred pentamers, and, Figures 39A to 39P show a library of stereoisomers up to and including the pentamer, from which other compounds within the scope of the invention can be prepared, without undue experimentation.

Example 18: NMR Evaluation of Pyrocyanidins

¹³C NMR spectroscopy was deemed a generally useful technique for the study of procyanidins, especially as the phenols usually provide good quality spectra, whereas proton NMR spectra are considerably broadened. The ¹³C NMR spectra

of oligomers yielded useful information for A or B ring substitution patterns, the relative stereochemistry of the C ring and in certain cases, the position of the interflavanoid linkages. Nonetheless, ¹H NMR spectra 5 yielded useful information.

Further, HOHAHA, makes use of the pulse technique to transfer magnetization of a first hydrogen to a second in a sequence to obtain cross peaks corresponding to alpha, beta, gamma or delta protons. COSY is a 2D-Fourier 10 transform NMR technique wherein vertical and horizontal axes provide ¹H chemical shift and 1D spectra; and a point of intersection provides a correlation between protons, whereby spin-spin couplings can be determined. HMQC spectra enhances the sensitivity of NMR spectra of nuclei; other 15 than protons and can reveal cross peaks from secondary and tertiary carbons to the respective protons. APT is a ¹³C technique used in determining the number of hydrogens present at a carbon. An even number of protons at a carbon will result in a positive signal, while an odd number of 20 protons at a carbon will result in a negative signal.

Thus ¹³C NMR, ¹H NMR, HOHAHA (homonuclear Hartmann-Hahn), HMQC (heteronuclear multiple quantum coherence), COSY (Homonuclear correlation spectroscopy), APT (attached proton test), and XHCORR (a variation on HMQC) spectroscopy were 25 used to elucidate the structures of the inventive compounds.

Method A. Monomer

All spectra were taken in deuterated methanol, at room temperature, at an approximate sample concentration of 10mg/mL. Spectra were taken on a Bruker 500 MHZ NMR, using 30 methanol as an internal standard.

Figures 44A-E represent the NMR spectra which were used to characterize the structure of the epicatechin

monomer. Figure 44A shows the ^1H and ^{13}C chemical shifts, in tabular form. Figures 44 B-E show ^1H , APT, XHCORR and COSY spectra for epicatechin.

Similarly, Figures 45A-F represent the NMR spectra which were used to characterize the structure of the catechin monomer. Figure 45A shows the ^1H and ^{13}C chemical shifts, in tabular form. Figures 44 B-F show ^1H , ^{13}C , APT, XHCORR and COSY spectra for catechin.

Method B. Dimers

10 All spectra were taken in 75% deuterated acetone in D_2O , using acetone as an internal standard, and an approximate sample concentration of 10mg/mL.

15 Figures 46A-G represent the spectra which were used to characterize the structure of the B2 dimer. Fig. 46A shows ^1H and ^{13}C chemical shifts, in tabular form. The terms T and B indicate the top half of the dimer and the bottom half of the dimer.

20 Figures 46B and C show the ^{13}C and APT spectra, respectively, taken on a Bruker 500 MHZ NMR, at room temperature.

Figures 46D-G show the ^1H , HMQC, COSY and HOHAHA, respectively, which were taken on AMZ-360 MHZ NMR at a -7°C. The COSY spectrum was taken using a gradient pulse.

25 Figures 47A-G represent the spectra which were used to characterize the structure of the B5 dimer. Figure 47A shows the ^{13}C and ^1H chemical shifts, in tabular form.

Figures 47B-D show the ^1H , ^{13}C and APT, respectively, which were taken on a Bruker 500 MHZ NMR, at room temperature.

30 Figure 47E shows the COSY spectrum, taken on an AMX-360, at room temperature, using a gradient pulse.

Figures 47F and G show the HMQC and HOHAHA, respectively, taken on an AMX-360 MHZ NMR, at room temperature.

5 Method C. Trimer - Epicatechin/Catechin

All spectra were taken in 75% deuterated acetone in D₂O, at -3°C using acetone as an internal standard, on an AMX-360 MHZ NMR, and an appropriate sample concentration of 10mg/mL.

10 Figures 48A-D represent the spectra which were used to characterize the structure of the epicatechin/catechin trimer. These figures show ¹H, COSY, HMQC and HOHAHA, respectively. The COSY spectrum was taken using a gradient pulse.

15 Method D. Trimer -All Epicatechin

All spectra were taken in 70% deuterated acetone in D₂O, at -1.8°C, using acetone as an internal standard, on an AMX-360 MHZ NMR, and an appropriate sample concentration of 10mg/mL.

20 Figures 49A-D represent the spectra which were used to characterize the structure of all epicatechin trimer. These figures show ¹H, COSY, HMQC and HOHAHA, respectively. The COSY spectrum was taken using a gradient pulse.

25 Example 19: Thiolysis of Procyanidins

In an effort to characterize the structure of procyanidins, benzyl mercaptan (BM) was reacted with catechin, epicatechin or dimers B2 and B5. Benzyl mercaptan, as well as phloroglucinol and thiophenol, can be 30 utilized in the hydrolysis (thiolysis) of procyanidins in an alcohol/acetic acid environment. Catechin, epicatechin or dimer (1:1 mixture of B2 and B5 dimers) (2.5mg) was dissolved

in 1.5mL ethanol, 100 μ l BM and 50 μ l acetic acid, and the vessel (Beckman amino acid analysis vessel) was evacuated and purged with nitrogen repeatedly until a final purge with nitrogen was followed by sealing the reaction vessel. The 5 reaction vessel was placed in a heat block at 95°C, and aliquots of the reaction were taken at 30, 60, 120 and 240 minutes. The relative fluorescence of each aliquot is shown in Figures 25A-C, representing epicatechin, catechin and dimers, respectively. Higher oligomers are similarly 10 thiolyzed.

Example 20: Thiolytic and Desulfurization of Dimers

Dimers B2 and B5 were hydrolyzed with benzylmercaptan by dissolving dimer (B2 or B5; 1.0 mg) in 600 μ l ethanol, 40 μ l BM and 20 μ l acetic acid. The mixture 15 was heated at 95°C for 4 hours under nitrogen in a Beckman Amino Acid Analysis vessel. Aliquots were removed for analysis by reverse-phase HPLC, and 75 μ l of each of ethanol Raney Nickel and gallic acid (10mg/mL) were added to the remaining reaction medium in a 2mL hypovial. The vessel was 20 purged under hydrogen, and occasionally shaken for 1 hour. The product was filtered through a 0.45 μ filter and analyzed by reverse-phase HPLC. Representative elution profiles are shown in Figures 26 A and B. Higher oligomers are similarly 25 desulfurized. This data suggests polymerization of epicatechin or catechin and therefore represents a synthetic route for preparation of inventive compounds.

Example 21: In vivo Activity of Pentamer in MDA MB 231 Nude Mouse Model

30 MDA-MB-231/LCC6 cell line. The cell line was grown in improved minimal essential medium (IMEM) containing 10% fetal bovine serum and maintained in a humidified, 5% CO₂ atmosphere at 37°C.

Mice. Female six to eight week old NCr nu/nu (athymic) mice were purchased through NCI and housed in an animal facility and maintained according to the regulations set forth by the United States Department of Agriculture, 5 and the American Association for the Accreditation of Laboratory Animal Care. Mice with tumors were weighed every other day, as well as weekly to determine appropriate drug dosing.

Tumor implantation. MDA-MD-231 prepared by tissue culture was diluted with IMEM to 3.3×10^6 cells/mL and 10 0.15mL (i.e. 0.5×10^6 cells) were injected subcutaneously between nipples 2 and 3 on each side of the mouse. Tumor volume was calculated by multiplying: length x width x height x 0.5. Tumor volumes over a treatment group were 15 averaged and Student's t test was used to calculate p values.

Sample preparation. Plasma samples were obtained by cardiac puncture and stored at -70°C with 15-20 mM EDTA for the purposes of blood chemistry determinations. No 20 differences were noted between the control group and experimental groups.

Fifteen nude mice previously infected with 500,000 cells subcutaneously with tumor cell line MDA-MB- 231, were randomly separated into three groups of 5 animals each and 25 treated by intraperitoneal injection with one of: (i) placebo containing vehicle alone (DMSO); (ii) 2mg/mouse of purified pentameric procyanidin extract as isolated in Example 14 method D in vehicle (DMSO); and (iii) 10mg/mouse purified pentameric procyanidin extract as isolated in 30 Example 14, method D in vehicle (DMSO).

The group (iii) mice died within approximately 48 to 72 hours after administration of the 10mg, whereas the

group (ii) mice appeared normal. The cause of death of the group (iii) mice was undetermined; and, cannot necessarily be attributed to the administration of inventive compounds. Nonetheless, 10mg was considered an upper limit with respect
5 to toxicity.

Treatment of groups (i) and (ii) was repeated once a week, and tumor growth was monitored for each experimental and control group. After two weeks of treatment, no signs of toxicity were observed in the mice of group (ii) and, the
10 dose administered to this group was incrementally increased by 1/2 log scale each subsequent week. The following Table represents the dosages administered during the treatment schedule for mice of group (ii):

	Week	Dose (mg/mouse)
15	1	2
	2	2
	3	4
	4	5
20	5	5
	6	5
	7	5

The results of treatment are shown in Figures 27A
25 and B and Table 10.

TABLE 10: IN VIVO ANTI-CANCER RESULTS

DAY	% SURVIVAL GROUP (i)	% SURVIVAL GROUP (ii)	% SURVIVAL GROUP (iii)
5	1	100	100
	2	100	100
	3	100	100
	4	100	100
	5	100	100
10	6	100	100
	7	100	100
	8	100	100
	9	100	100
	10	100	100
15	11	100	100
	12	100	100
	13	100	100
	14	100	100
	15	100	100
20	16	100	100
	17	100	100
	18	100	100
	19	100	100
	20	100	100
25	21	100	100
	22	75	100
	23	75	100
	24	75	100
	25	75	100

DAY	% SURVIVAL GROUP (i)	% SURVIVAL GROUP (ii)	% SURVIVAL GROUP (iii)
5	26	75	100
	27	75	100
	28	75	100
	29	50	100
	30	50	100
	31	50	100
	32	50	100
10	33	50	100
	34	50	100
	35	50	100
	36	25	100
	37	25	100
	38	25	100
	39	25	100
15	40	25	100
	41	25	100
	42	25	100
	43	25	80
	44	25	80
	45	25	80
	46	25	80
20	47	25	80
	48	25	80
	49	25	80
	50	25	60
	51	25	60

DAY	% SURVIVAL GROUP (i)	% SURVIVAL GROUP (ii)	% SURVIVAL GROUP (iii)
5	52	25	60
	53	25	60
	54	25	60
	55	25	60
	56	25	60
	57	0	40
	58		40
	59		40
	60		40
	61		40
10	62		40
	63		40
	64		40

15 These results demonstrate that the inventive fractions and the inventive compounds indeed have utility in antineoplastic compositions, and are not toxic in low to medium dosages, with toxicity in higher dosages able to be determined without undue experimentation.

20 Example 22: Antimicrobial Activity of Cocoa Extracts

Method A:

25 A study was conducted to evaluate the antimicrobial activity of crude procyanidin extracts from cocoa beans against a variety of microorganisms important in food spoilage or pathogenesis. The cocoa extracts from Example 2, method A were used in the study. An agar medium appropriate for the growth of each test culture (99mL) was seeded with 1 mL of each cell culture suspension in 0.45% saline (final population 10^2 - 10^4 cfu/mL), and poured into

petri dishes. Wells were cut into hardened agar with a #2 cork borer (5mm diameter). The plates were refrigerated at 4°C overnight, to allow for diffusion of the extract into the agar, and subsequently incubated at an appropriate

- 5 growth temperature for the test organism. The results were as follows:

Sample Zone of Inhibition (mm)

	Extract Concentration (mg/mL)	<i>B. sphaericus</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>
10	0	N1	N1	N1	N1	N1
	25	N1	12	N1	11	N1
	250	12	20	19	19	11
	500	14	21	21	21	13

N1 = no inhibition

- Antimicrobial activity of purified procyanidin
 20 extracts from cocoa beans was demonstrated in another study using the well diffusion assay described above (in Method A) with *Staphylococcus aureus* as the test culture. The results were as follows:

- cocoa extracts: 10mg/100 μL decaffeinated/
 25 detheobrominated acetone extract
 as in Example 13, method A
- 30 10mg/100 μL dimer (99% pure)
 as in Example 14, method D
- 35 10mg/100 μL tetramer (95% pure) as in
 Example 14, method D
- 10mg/100 μL hexamer (88% pure) as in
 Example 14, method D
- 10mg/100 μL octamer/nanomer (92% pure)
 as in Example 14, method D

10mg/100 μ L nanomer & higher (87% pure)
as in Example 14, method D

<u>Sample Zone of Inhibition (mm)</u>		
5	0.45% saline	0
	Dimer	33
	Tetramer	27
	Hexamer	24
10	0.45% saline	0
	Octamer	22
	Nanomer	20
	Decaff./detheo.	26

15 Method B:

Crude procyanidin extract as in Example 2, method 2 was added in varying concentrations to TSB (Trypticase Soy Broth) with phenol red (0.08g/L), The TSB were inoculated 20 with cultures of *Salmonella enteritidis* or *S. newport* (10^5 cfu/mL), and were incubated for 18 hours at 35°C. The results were as follows:

		<u><i>S. enteritidis</i></u>	<u><i>S. Newport</i></u>
25	0mg/mL	+	+
	50	+	+
	100	+	+
	250	+	-
	500	-	-
30	750	-	-

where + = outgrowth, and - = no growth, as evidenced by the change in broth culture from red to yellow with acid 35 production. Confirmation of inhibition was made by plating from TSB tubes onto XLD plates.

This Example demonstrates that the inventive compounds are useful in food preparation and preservation.

This Example further demonstrates that gram 40 negative and gram positive bacterial growth can be inhibited by the inventive compounds. From this, the inventive

compounds can be used to inhibit *Helicobacter pylori*. *Helicobacter pylori* has been implicated in causing gastric ulcers and stomach cancer. Accordingly, the inventive compounds can be used to treat or prevent these and other 5 maladies of bacterial origin. Suitable routes of administration, dosages, and formulations can be determined without undue experimentation considering factors well known in the art such as the malady, and the age, weight, sex, general health of the subject.

10 Example 23: Halogen-free Analytical Separation of Extract

Procyanidins obtained from Example 2 were partially purified by Analytical Separation by Halogen-free Normal Phase Chromatography on 100Å Supelcosil LC-Si 5 μ m 15 (250 x 4.6mm), at a flow rate of 1.0mL/min, and a column temperature of 37°C. Separations were aided by a linear gradient under the following conditions: (time, %A, %B); (0, 82, 14); (30, 67.6, 28.4); (60, 46, 50). Mobile phase composition was A = 30/70 % diethyl ether/Toluene; B = 20 Methanol; and C = acetic acid/water (1:1). Components were detected by UV at 280nm. A representative elution profile is shown in Figure 28.

Example 24: Effect of Pore Size of Stationary Phase for Normal Phase HPLC Separation of Procyanidins

25 To improve the separation of procyanidins, the use of a larger pore size of the silica stationary phase was investigated. Separations were effected on Silica-300, 5 μ m, 300Å (250 x 2.0mm), or, in the alternative, on Silica-1000, 30 5 μ m, 1000Å (250 x 2.0mm). A linear gradient was employed as mobile phase composition was: A = Dichloromethane; B = Methanol; and C = acetic acid/water (1:1). Components were detected by fluorescence, wherein λ_{ex} = 276nm and λ_{em} = - 316nm, by UV detector at 280nm. The flow rate was

1.0mL/min, and the oven temperature was 37°C. A representative chromatogram from three different columns (100Å pore size, from Example 13, Method D) is shown in Figure 29. This shows effective pore size for separation of 5 procyanidins.

Example 25: Obtaining Desired Procyaniidins Via Manipulating Fermentation

Microbial strains representative of the succession 10 associated with cocoa fermentation were selected from the M&M/Mars cocoa culture collection. The following isolates were used:

15 *Acetobacter aceti* ATCC 15973
 Lactobacillus sp. (BH 42)
 Candida krusei (BA 15)
 Saccharomyces cerevisiae (BA 13)
 Bacillus cereus (BE 35)
 Bacillus sphaericus (ME 12)

20 Each strain was transferred from stock culture to fresh media. The yeasts and *Acetobacter* were incubated 72 hours at 26°C and the bacilli and *Lactobacillus* were incubated 48 hours at 37°C. The slants were harvested with 5mL phosphate buffer prior to use.

25 Cocoa beans were harvested from fresh pods and the pulp and testa removed. The beans were sterilized with hydrogen peroxide (35%) for 20 seconds, followed by treatment with catalase until cessation of bubbling. The beans were rinsed twice with sterile water and the process 30 repeated. The beans were divided into glass jars and processed according to the regimens detailed in the following Table:

Water	Ethanol/acid	Fermentation infusate	Model Fermentation
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daily transfer to fresh water	daily transfer to solutions of alcohol and acid corresponding to levels determined at each stage of a model pulp fermentation	daily transfer to fermented pulp pasteurized on each successive day of fermentation	bench scale model fermentation in sterile pulp coinoculated with test strains
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The bench scale fermentation was performed in duplicate. All treatments were incubated as indicated below:

- Day 1: 26°C
 10 Day 2: 26°C to 50°C
 Day 3: 50°C
 Day 4: 45°C
 Day 5: 40°C

- 15 The model fermentation was monitored over the duration of the study by plate counts to assess the microbial population and HPLC analysis of the fermentation medium for the production of microbial metabolites. After treatment, the beans were dried under a laminar flow hood to a water activity of 0.64 and were roasted at 66°C for 15 min. Samples were prepared for procyanidin analysis. Three beans per treatment were ground and defatted with hexane, followed by extraction with an acetone:water:acetic acid (70:29.5:0.5%) solution. The acetone solution extract was filtered into vials and polyphenol levels were quantified by normal phase HPLC as in Example 13, method B. The remaining beans were ground and tasted. The cultural and analytical profiles of the model bench-top fermentation process is shown in Figures 30A - C. The procyanidin profiles of cocoa beans subjected to various fermentation treatments is shown in Figure 30D.
- 20 25 30

This Example demonstrates that the invention need not be limited to any particular cocoa genotype; and, that by manipulating fermentation, the levels of procyanidins produced by a particular *Theobroma* or *Herrania* species or 5 their inter or intra species specific crosses thereof can be modulated, e.g., enhanced.

The following Table shows procyanidin levels determined in specimens which are representative of the *Theobroma* genus and their inter and intra species specific 10 crosses. Samples were prepared as in Examples 1 and 2 (methods 1 and 2), and analyzed as in Examples 13, method B. This data illustrates that the extracts containing the inventive compounds are found in *Theobroma* and *Herrania* species, and their intra and inter species specific crosses.

Theobroma Species Proganicidin Levels
 ppm ($\mu\text{g/g}$) in defatted powder

SAMPLE	Number	Oligomer										Total
		Dime	Triole	Trioleine	Hepaone	Hepaone	Octone	Nonenone	Decanone	Undecanone		
<i>T. grandiflorum</i> x <i>T. obovatum</i> 1'	3822	3442	5384	4074	3146	2080	850	421	348	198	Ir*	23,765
<i>T. grandiflorum</i> x <i>T. obovatum</i> 2'	3003	4098	5411	3983	2931	1914	1050	577	356	198	Ir	23,561
<i>T. grandiflorum</i> x <i>T. obovatum</i> 3A'	4980	4980	7556	5341	4008	2576	1075	598	301	144	Ir	31,569
<i>T. grandiflorum</i> x <i>T. obovatum</i> 3B'	3880	4498	6488	4930	3706	2560	1208	593	323	174	Ir	28,350
<i>T. grandiflorum</i> x <i>T. obovatum</i> 4'	2647	3591	5328	4240	3304	2380	1506	815	506	249	Ir	24,566
<i>T. grandiflorum</i> x <i>T. obovatum</i> 6'	2754	3855	5299	3872	2994	1990	1159	629	359	196	Ir	23,194
<i>T. grandiflorum</i> x <i>T. obovatum</i> S/N'	3212	4134	7608	4736	3530	2274	936	446	278	126	ND*	23,750
<i>T. obovatum</i> 1'	3682	5683	9512	5358	3858	2454	1207	640	302	144	ND	32,820
<i>T. grandiflorum</i> TEEFE ²	2608	2178	3050	2704	2241	1586	900	484	301	148	Ir	16,240
<i>T. Grandiflorum</i> TEEFE x <i>T. grandiflorum</i> ¹	4773	4056	5289	4748	3804	2444	998	737	335	156	Ir	27,380
<i>T. grandiflorum</i> x <i>T. subincanum</i> ¹	4752	3336	4916	3900	3064	2039	782	435	380	228	ND	23,832
<i>T. obovatum</i> x <i>T. subincanum</i> ¹	3379	3802	5836	3940	2868	1807	814	427	271	136	Ir	23,280
<i>T. speciosum</i> x <i>T. sylvesteris</i> ¹	902	346	1350	217	152	120	60	Ir	Ir	ND	ND	3,147
<i>T. microcarpum</i> ²	5694	3230	2766	1490	822	356	141	Ir	ND	ND	ND	14,519
<i>T. cacao</i> , SIAL 659, 10	2,929	10,072	10,106	7768	5311	3242	1311	626	422	146	Ir	60,753
<i>T. cacao</i> , SIAL 659, 11	21,088	9762	9119	7094	4774	2906	1364	608	361	176	Ir	57,252
<i>T. cacao</i> , SIAL 659, 148	20,887	9892	9474	7337	4906	2929	1334	632	412	302	Ir	56,165
<i>T. cacao</i> , SIAL 659, 196	9552	5780	5062	3360	2140	1160	464	254	138	Ir	ND	27,910
<i>T. cacao</i> , SIAL 659, 1120	6581	4685	4070	2527	1628	888	326	166	123	Ir	ND	22,974

*ND = none detected¹ sample designated CPATU

*Ir = trace (< 50/ $\mu\text{g/g}$) sample designated ERJON

Example 26: Effect of Procyanidins on NO
 Method A.

The purpose of this study is to establish the
5 relationship between procyanidins (as in Example 14, method
D) and NO, which is known to induce cerebral vascular
dilation. The effects of monomers and higher oligomers, in
concentrations ranging from 100 μ g/mL to 0.1 μ g/mL, on the
production of nitrates (the catabolites of NO), from HUVEC
10 (human umbilical vein endothelial cells) is evaluated.
HUVEC (from Clonetics) is investigated in the presence or
absence of each procyandin for 24 to 48 hours. At the end
of the experiments, the supernatants are collected and the
nitrate content determined by colorimetric assay. In
15 separate experiments, HUVEC is incubated with acetylcholine,
which is known to induce NO production, in the presence or
absence of procyanidins for 24 to 48 hours. At the end of
the experiments, the supernatants are collected and nitrate
content is determined by colorimetric assay. The role of NO
20 is ascertained by the addition of nitroarginine or (1)-N-
methyl arginine, which are specific blockers of NO synthase.

Method B. Vasorelaxation of Phenylephrine-Induced
Contracted Rat Artery

The effects of each of the procyanidins (100 μ g/mL
25 to 0.1 μ g/mL on the rat artery is the target for study of
vasorelaxation of phenylephrine-induced contracted rat
artery. Isolated rat artery is incubated in the presence or
absence of procyanidins (as in Example 14, method D) and
alteration of the muscular tone is assessed by visual
30 inspection. Both contraction or relaxation of the rat
artery is determined. Then, using other organs,
precontraction of the isolated rat artery is induced upon

addition of epinephrine. Once the contraction is stabilized, procyanidins are added and contraction or relaxation of the rat artery is determined. The role of NO is ascertained by the addition of nitroarginine or (1)-N-methyl arginine. The acetylcholine-induced relaxation of NO, as it is effected by phenylephrine-precontracted rat aorta is shown in Figure 31.

Method C. Induction of Hypotension in the Rat

This method is directed to the effect of each 10 procyanidin (as in Example 14, method D) on blood pressure. Rats are instrumented in order to monitor systolic and diastolic blood pressure. Each of the procyanidins are injected intravenously (dosage range = 100 - 0.1 μ g/kg), and alteration of blood pressure is assessed. In addition, the 15 effect of each procyanidin on the alteration of blood pressure evoked by epinephrine is determined. The role of NO is ascertained by the addition of nitroarginine or (1)-N-methyl arginine.

These studies, together with next Example, 20 illustrate that the inventive compounds are useful in modulating vasodilation, and are further useful with respect to modulating blood pressure or addressing coronary conditions, and migraine headache conditions.

Example 27: Effects of Cocoa Polyphenols on Satiety

25 Using blood glucose levels as an indicator for the signal events which occur *in vivo* for the regulation of appetite and satiety, a series of simple experiments were conducted using a healthy male adult volunteer age 48 to determine whether cocoa polyphenols would modulate glucose 30 levels. Cocoa polyphenols were partially purified from Brazilian cocoa beans according to the methods described by

Clapperton et al. (1992). This material contained no caffeine or theobromine. Fasting blood glucose levels were analyzed on a timed basis after ingestion of 10 fl. oz of Dexicola 75 (caffeine free) Glucose tolerance test beverage (Curtin Matheson 091-421) with and without 75mg cocoa polyphenols. This level of polyphenols represented 0.1% of the total glucose of the test beverage and reflected the approximate amount that would be present in a standard 100g chocolate bar. Blood glucose levels were determined by using the Accu-Chek III blood glucose monitoring system (Boehringer Mannheim Corporation). Blood glucose levels were measured before ingestion of test beverage, and after ingestion of the test beverage at the following timed intervals: 15, 30, 45, 60, 75, 90, 120 and 180 minutes.

Before the start of each glucose tolerance test, high and low glucose level controls were determined. Each glucose tolerance test was performed in duplicate. A control test solution containing 75mg cocoa polyphenols dissolved in 10 fl. oz. distilled water (no glucose) was also performed.

Table 11 below lists the dates and control values obtained for each glucose tolerance experiment performed in this study. Figure 32 represents plots of the average values with standard deviations of blood glucose levels obtained throughout a three hour time course. It is readily apparent that there is a substantial increase in blood sugar levels was obtained after ingestion of a test mixture containing cocoa polyphenols. The difference between the two principal glucose tolerance profiles could not be resolved by the profile obtained after ingestion of a solution of cocoa polyphenols alone. The addition of cocoa polyphenols to the glucose test beverage raised the glucose tolerance profile significantly. This elevation in blood

glucose levels is within the range considered to be mildly diabetic, even though the typical glucose tolerance profile was considered to be normal (Davidson, I. et al., Eds. Todd - Sandford Clinical Diagnosis by Laboratory Methods 14th edition; W.B. Saunders Co.; Philadelphia, PA 1969 Ch. 10, pp. 550-9). This suggests that the difference in additional glucose was released to the bloodstream, from the glycogen stores, as a result of the inventive compounds. Thus, the inventive compounds can be used to modulate blood glucose levels when in the presence of sugars.

Table 11. Glucose Tolerance Test Dates and Control Results

WEEK	DESCRIPTION	HIGH CONTROL ^a	LOW CONTROL ^b
15	0 Glucose Tolerance	265 mg/dL	53 mg/dL
	1 Glucose Tolerance with 0.1% polyphenols	310	68
	2 Glucose Tolerance	315	66
	4 Glucose Tolerance with 0.1% polyphenols	325	65
	5 0.1% polyphenols	321	66

20 a = Expected range: 253 - 373mg/dL
 b = Expected range: 50-80mg/dL

The subject also experienced a facial flush (erythema) and lightheadedness following ingestion of the inventive compounds, indicating modulation of vasodilation.

- The data presented in Tables 12 and 13 illustrates
5 the fact that extracts of the invention pertaining to cocoa raw materials and commercial chocolates, and inventive compounds contained therein can be used as a vehicle for pharmaceutical, veterinary and food science preparations and applications.

Procyanidin Levels in Commercial Chocolates
µg/g

Sample	Monomers	Dimers	Trimmers	Tetramers	Pentamers	Hexamers	Heptamers and Higher	Total
Brand 1	366	166	113	59	56	23	18	801
Brand 2	344	163	111	45	48	ND*	ND	711
Brand 3	316	181	100	41	40	7	ND	685
Brand 4	310	122	71	27	28	5	ND	563
Brand 5	259	135	90	46	29	ND	ND	559
Brand 6	308	139	91	57	47	14	ND	656
Brand 7	196	98	81	58	54	19	ND	506
Brand 8	716	472	302	170	117	18	ND	1,795
Brand 9	1,185	951	633	298	173	25	21	3,286
Brand 10	1,798	1,081	590	342	307	93	ND	4,211
Brand 11	1,101	746	646	372	347	130	75	3,417
Brand 12	787	335	160	20	10	8	ND	1,320

ND* = None detected.

Procyanidin Levels in Cocoa Raw Materials

µg/g

Sample	Monomers	Dimers	Trimmers	Tetramers	Pentamers	Hexamers	Heptamers and Higher	Total
Unfermented	13,440	6,425	6,401	5,292	4,236	3,203	5,913	44,910
Fermented	2,695	1,538	1,362	740	470	301	277	7,383
Roasted	2,656	1,597	921	337	164	ND*	ND	5,675
Choc. Liquor	2,805	1,446	881	442	184	108	ND	5,866
Cocoa Hulls	114	53	14	ND	ND	ND	ND	181
Cocoa Powder 1% Fat	506	287	287	112	ND	ND	ND	915
Cocoa Powder 11% Fat	1,523	1,224	680	46	ND	ND	ND	3,473
Red Dutch Cocoa Powder, PH 7.4, 11% fat	1,222	483	103	ND	ND	ND	ND	1,808
Red Dutch Cocoa Powder, PH 8.2, 23% fat	168	144	60	ND	ND	ND	ND	372

ND* = None detected.

**Example 28: The Effect of Procyanidins on
cyclooxygenase 1 & 2**

The effect of procyanidins on cyclooxygenase 1 & 2 (COX1/COX2) activities was assessed by incubating the enzymes, derived from ram seminal vesicle and sheep placenta, respectively, with arachidonic acid ($5\mu M$) for 10 minutes at room temperature, in the presence of varying concentrations of procyanidin solutions containing monomer to decamer and procyanidin mixture. Turnover was assessed by using PGE2 EIA kits from Interchim (France). Indomethacin was used as a reference compound. The results are presented in the following Table, wherein the IC_{50} values are expressed in units of μM (except for S11, which represents a procyanidin mixture prepared from Example 13, Method A and where the samples S1 to S10 represent sequentially procyanidin oligomers (monomer through decamer) as in Example 14, Method D, and IC_{50} is expressed in units of mg/mL).

20

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SAMPLE #	IC ₅₀ COX-1 (*)	IC ₅₀ COX-2 (*)	RATIO IC ₅₀ COX2/COX1
5	1	0.074	0.197
	2	0.115	0.444
	3	0.258	0.763
	4	0.154	3.73
	5	0.787	3.16
	6	1.14	1.99
10	7	1.89	4.06
	8	2.25	7.2
	9	2.58	2.08
	10	3.65	3.16
	11	0.0487	0.0741
Indomethoacin		0.599	13.5
			22.54

15 (*) expressed as mM with the exception of sample 11,
which is mg/mL.

20 The results of the inhibition studies are
presented in Figures 33 A and B, which shows the effects of
Indomethacin on COX1 and COX2 activities. Figures 34 A and
B shows the correlation between the degree of polymerization
of the procyanidin and IC₅₀ with COX1 and COX2; Figure 35
shows the correlation between IC₅₀ values on COX1 and COX2.
And, Figures 36 A through Y show the IC₅₀ values of each
25 sample (S1 - S11) with COX1 and COX2.

These results indicate that the inventive
compounds have analgesic, anti-coagulant, and anti-
inflammatory utilities. Further, COX2 has been linked to
colon cancer. Inhibition of COX2 activity by the inventive

compounds illustrates a plausible mechanism by which the inventive compounds have antineoplastic activity against colon cancer.

COX1 and COX2 are also implicated in the synthesis of prostaglandins. Thus, the results in this Example also indicate that the inventive compounds can modulate renal functions, immune responses, fever, pain, mitogenesis, apoptosis, prostaglandin synthesis, ulceration (e.g., gastric), and reproduction. Note that modulation of renal function can affect blood pressure; again implicating the inventive compounds in modulating blood pressure, vasodilation, and coronary conditions (e.g., modulation of angiotensin, bradykinin).

Reference is made to Seibert et al., PNAS USA 91:12013-12017 (December, 1994), Mitchell et al., PNAS USA 90:11693-11697 (December 1994), Dewitt et al., Cell 83:345-348 (November 3, 1995), Langenbach et al., Cell 83:483-92 (November 3, 1995) and Sujii et al., Cell 83:493-501 (November 3, 1995), Morham et al., Cell 83:473-82 (November 3, 1995).

Reference is further made to Examples 9, 26, and 27. In Example 9, the anti-oxidant activity of inventive compounds is shown. In Example 26, the effect on NO is demonstrated. And, Example 27 provides evidence of a facial vasodilation. From the results in this Example, in combination with Examples 9, 26 and 27, the inventive compounds can modulate free radical mechanisms driving physiological effects. Similarly, lipoxygenase mediated free radical type reactions biochemically directed toward leukotriene synthesis can be modulated by the inventive

compounds, thus affecting subsequent physiological effects (e.g., inflammation, immune response, coronary conditions, carcinogenic mechanisms, fever, pain, ulceration).

Thus, in addition to having analgesic properties,
5 there may also be a synergistic effect by the inventive compounds when administered with other analgesics.
Likewise, in addition to having antineoplastic properties,
there may also be a synergistic effect by the inventive
compounds when administered with other antineoplastic
10 agents.

Example 29:Circular Dichroism/Study of Procyanidins

CD studies were undertaken in an effort to elucidate the structure of purified procyanidins as in Example 14, Method D. The spectra were collected at 25°C
15 using CD spectrum software AVIV 60DS V4.1f.

Samples were scanned from 300nm to 185nm, every 1.00nm, at 1.50nm bandwidth. Representative CD spectra are shown in Figures 43A through G, which show the CD spectra of dimer through octamer.

20 These results are indicative of the helical nature of the inventive compounds.

From the foregoing, it is clear that the extract and cocoa polyphenols, particularly the inventive compounds, as well as the compositions, methods, and kits, of the
25 invention have significant and numerous utilities.

The antineoplastic utility is clearly demonstrated by the *in vivo* and *in vitro* data herein and shows that

inventive compounds can be used instead of or in conjunction with conventional antineoplastic agents.

The inventive compounds have antioxidant activity like that of BHT and BHA, as well as oxidative stability.

5 Thus, the invention can be employed in place of or in conjunction with BHT or BHA in known utilities of BHA and BHT, such as an antioxidant, for instance, an antioxidant; food additive.

10 The invention can also be employed in place of or in conjunction with topoisomerase II-inhibitors in the presently known utilities therefor.

15 The inventive compounds can be used in food preservation or preparation, as well as in preventing or treating maladies of bacterial origin. Simply the inventive compoundss can be used as an antimicrobial.

20 The inventive compounds can also be used as a cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase, or blood or in vivo glucose modulator, and are thus useful for treatment or prevention or modulation of pain, fever, inflammation coronary conditions, ulceration, carcinogenic mechanisms, vasodilation, as well as an analgesic, anti-coagulant anti-inflammatory and an immune response modulator.

25 Further, the invention comprehends the use of the compounds or extracts as a vehicle for pharmaceutical preparations. Accordingly, there are many compositions and methods envisioned by the invention. For instance, antioxidant or preservative compositions, topoisomerase II-inhibiting compositions, methods for preserving food or any

desired item such as from oxidation, and methods for inhibiting topoisomerase II. The compositions can comprise the inventive compounds. The methods can comprise contacting the food, item or topoisomerase II with the 5 respective composition or with the inventive compounds. Other compositions, methods and embodiments of the invention are apparent from the foregoing.

In this regard, it is mentioned that the invention is from an edible source and, that the activity *in vitro* can 10 demonstrate at least some activity *in vivo*; and from the *in vitro* and *in vivo* data herein, doses, routes of administration, and formulations can be obtained without undue experimentation.

Having thus described in detail the preferred 15 embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above descriptions as many apparent variations thereof are possible without departing from the spirit or scope of the 20 present invention.

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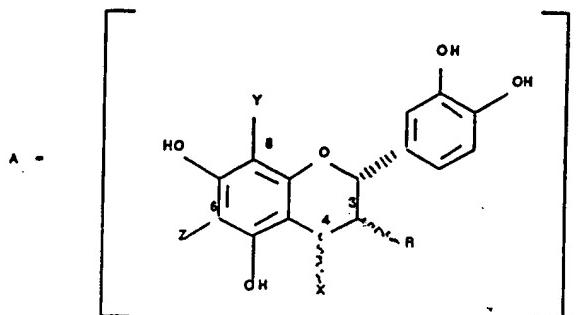
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WHAT IS CLAIMED IS:

1. A compound of the formula:



wherein:

n is an integer from 3 to 12, such that there is a first monomeric unit A, and a plurality of other monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

position 4 is alpha or beta stereochemistry;

X, Y and Z represent positions for bonding between monomeric units, with the provisos that as to the first monomeric unit, bonding of another monomeric unit thereto is at position 4 and Y = Z = hydrogen, and, that when not for bonding monomeric units, X, Y and Z are hydrogen or Z, Y are sugar and X is hydrogen, or X is alpha or beta sugar and Z + Y are hydrogen, or combinations thereof; and

the sugar can be optionally substituted with a phenolic moiety via an ester bond.

2. The compound of claim 1 wherein n is 5.
3. The compound of claim 1 wherein the sugar is selected from the group consisting essentially of glucose, galactose, xylose, rhamnose and arabinose.
4. An antineoplastic composition comprising a compound of claim 1 and a carrier or diluent.
5. An antineoplastic composition comprising a compound of claim 2 and a carrier or diluent.
6. An antioxidant composition comprising a compound of claim 1 and a carrier or diluent.
7. An antioxidant composition comprising a compound of claim 2 and a carrier or diluent.
8. An antimicrobial composition comprising a compound of claim 1 and a carrier or diluent.
9. An antimicrobial composition comprising a compound of claim 2 and a carrier or diluent.
10. A cyclo-oxygenase and/or lipoxygenase modulator composition comprising a compound of claim 1 and a carrier or diluent.
11. A cyclo-oxygenase and/or lipoxygenase modulator composition comprising a compound of claim 2 and a carrier or diluent.

12. An NO or NO-synthase modulating composition comprising a compound of claim 1 and a carrier or diluent.

13. An NO or NO-synthase modulating composition comprising a compound of claim 2 and a carrier or diluent.

14. An *in vivo* glucose-modulating composition comprising a compound of claim 1 and a carrier or diluent.

15. An *in vivo* glucose-modulating composition comprising a compound of claim 2 and a carrier diluent.

16. A method for treating a subject in need of treatment with an antineoplastic agent comprising administering to the subject an antineoplastic composition of claim 4.

17. A method for treating a subject in need of treatment with an antineoplastic agent comprising administering to the subject an antineoplastic composition of claim 5.

18. A method for treating a subject in need of treatment with an antioxidant agent comprising administering to the subject an antioxidant composition of a compound of claim 6.

19. A method for treating a subject in need of treatment with an antioxidant agent comprising administering to the subject an antioxidant composition of a compound of claim 7.

20. A method for treating a subject in need of treatment with an antimicrobial agent comprising

administering to the subject an antimicrobial composition of a compound of claim 8.

21. A method for treating a subject in need of treatment with an antimicrobial agent comprising administering to the subject an antimicrobial composition of claim 9.

22. A method for treating a subject in need of treatment with a cyclo-oxygenase or lipoxygenase modulating agent comprising administering to the subject a composition of claim 10.

23. A method for treating a subject in need of treatment with a cyclo-oxygenase or lipoxygenase modulating agent comprising administering to the patient a composition of claim 11.

24. A method for treating a subject in need of treatment with a NO or NO-synthase modulating agent comprising administering to the subject a composition of claim 12.

25. A method for treating a subject in need of treatment with a NO-modulating agent comprising administering to the subject a NO or NO-synthase modulating composition of claim 12.

26. A method for treating a subject in need of treatment with a glucose-modulating agent comprising administering to the subject a composition of claim 14.

27. A method for treating a subject in need of treatment with a glucose-modulating agent comprising administering to the subject a composition of claim 15.

28. An antioxidant or preservative composition comprising a compound as claimed in claim 1 and a diluent.

29. An antioxidant or preservative composition comprising a compound as claimed in claim 2 and a diluent.

30. A topoisomerase II-inhibiting composition comprising a compound as claimed in claim 1 and a carrier or diluent.

31. A topoisomerase II-inhibiting composition comprising a compound as claimed in claim 2 and a carrier or diluent.

32. A method for preserving or protecting a desired item from oxidation comprising contacting the item with a composition as claimed in claim 28.

33. A method for inhibiting topoisomerase II which comprises contacting topoisomerase II with a composition as claimed in claim 30.

34. A carrier or vehicle for a pharmaceutical comprising a compound as claimed in claim 1.

35. A carrier or vehicle for a pharmaceutical comprising a cocoa extract.

36. A kit for a composition of claim 4 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

37. A kit for a composition of claim 6 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

38. A kit for a composition of claim 8 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

39. A kit for a composition of claim 10 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

40. A kit for a composition of claim 12 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

41. A kit for a composition of claim 14 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

42. A kit for a composition of claim 30 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

43. A kit for a composition of claim 28 comprising the compound and the diluent separately packaged, and optionally instructions for admixture or use.

44. A substantially pure *Theobroma* or *Herrania* species or inter or their intra species specific crosses thereof extract comprising polyphenols comprising oligomers 3 through 12.

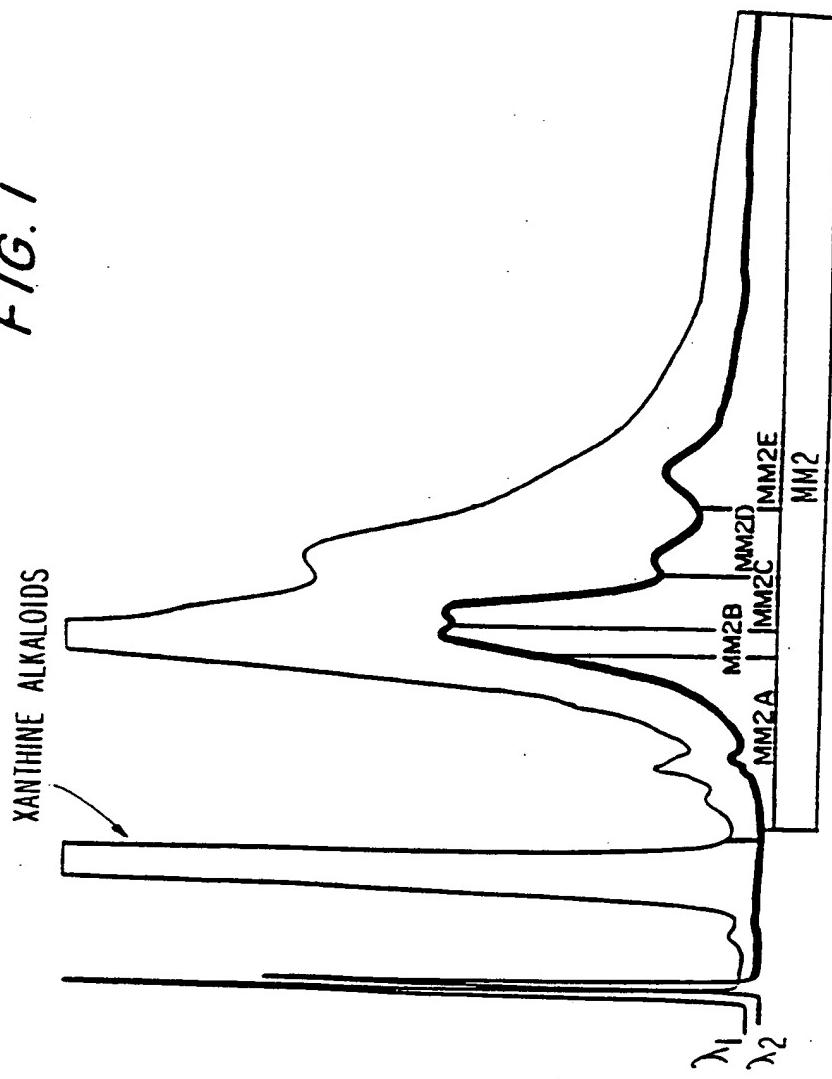
45. A compound of claim 1 having a structure giving an NMR spectra as set forth in Figure 48A-D.

46. A compound of claim 1 having a structure giving an NMR spectra as set forth in Figure 49A-D.

47. A method for enhancing the concentration levels and distribution of cocoa procyanidins in cocoa beans by manipulating fermentation conditions.

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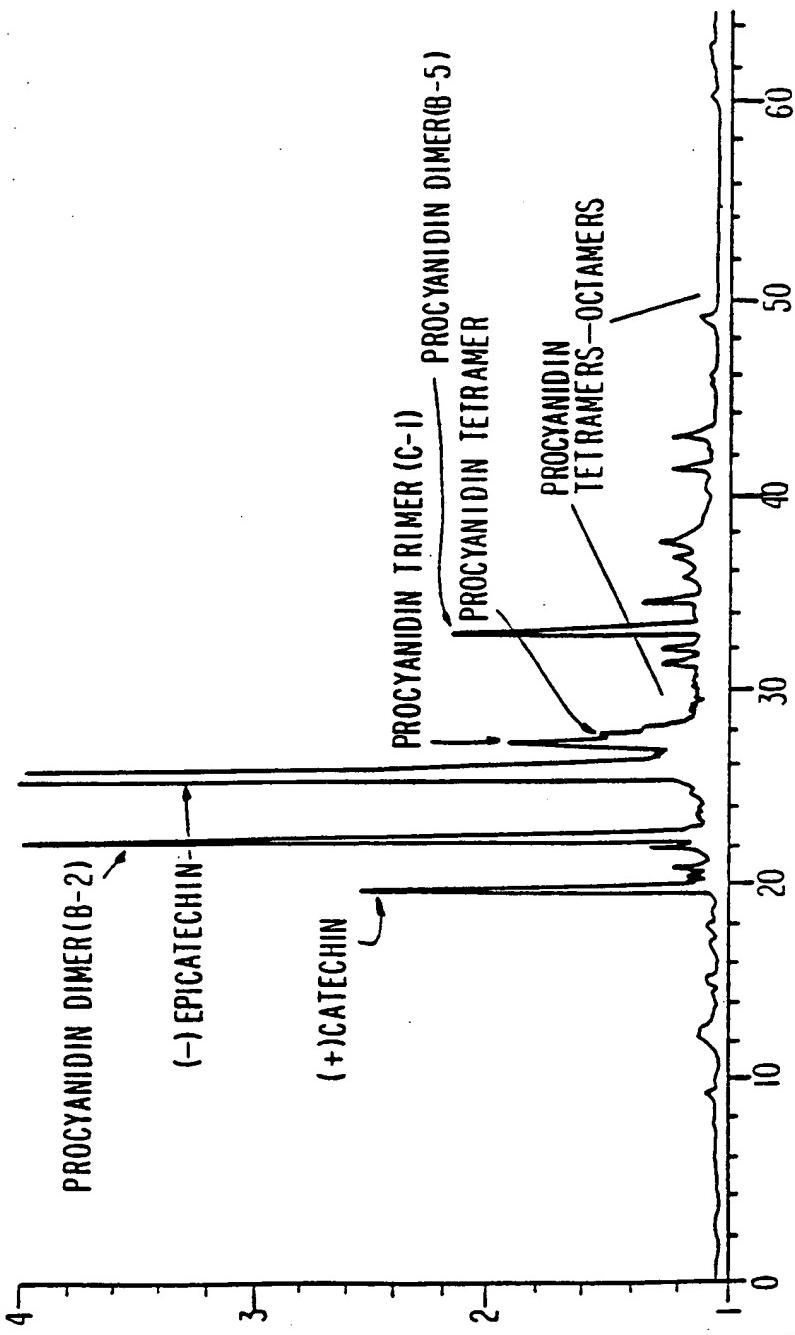
FIG. 1



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FIG. 2A

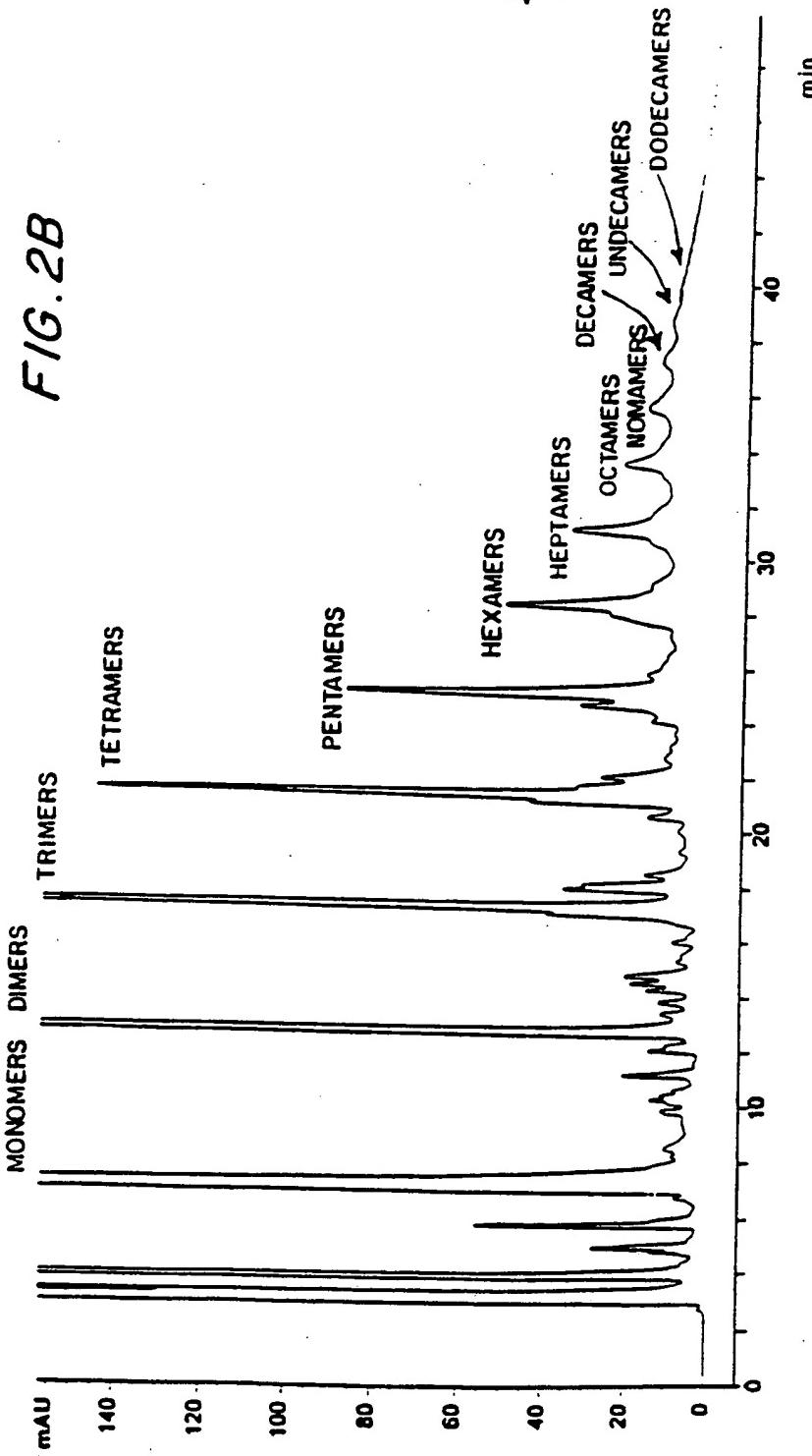


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FIG. 2B



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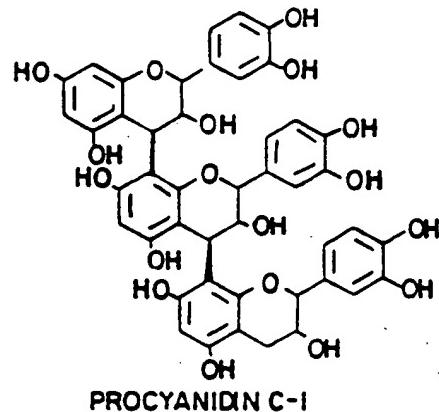
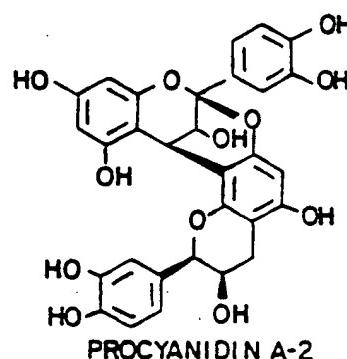
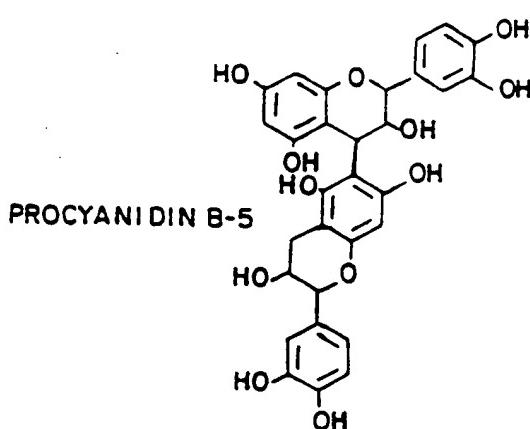
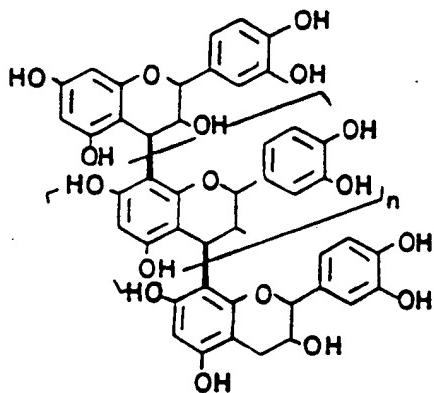
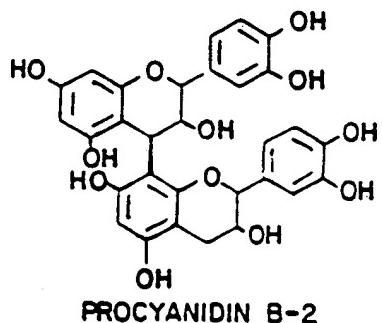
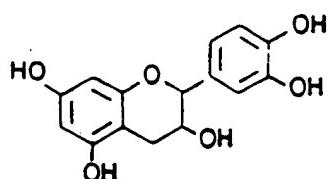
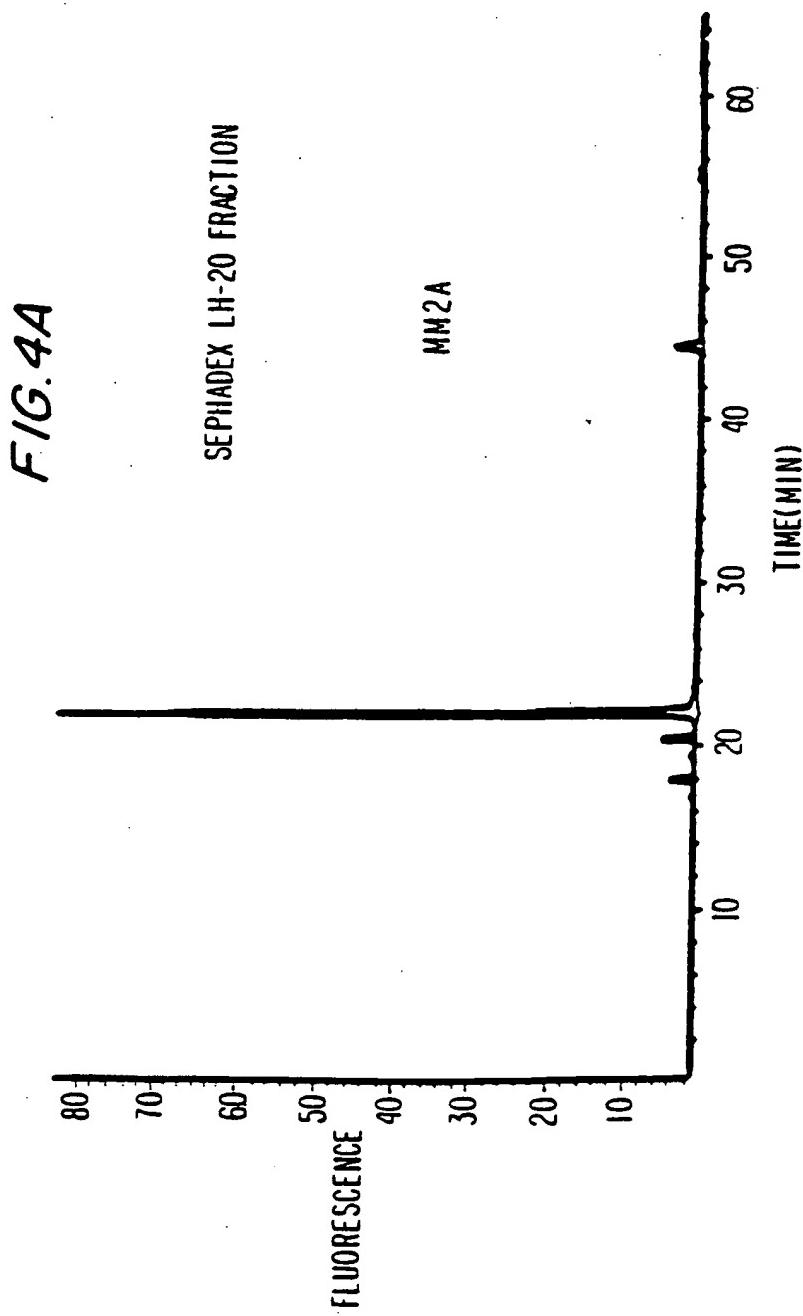


FIG. 3

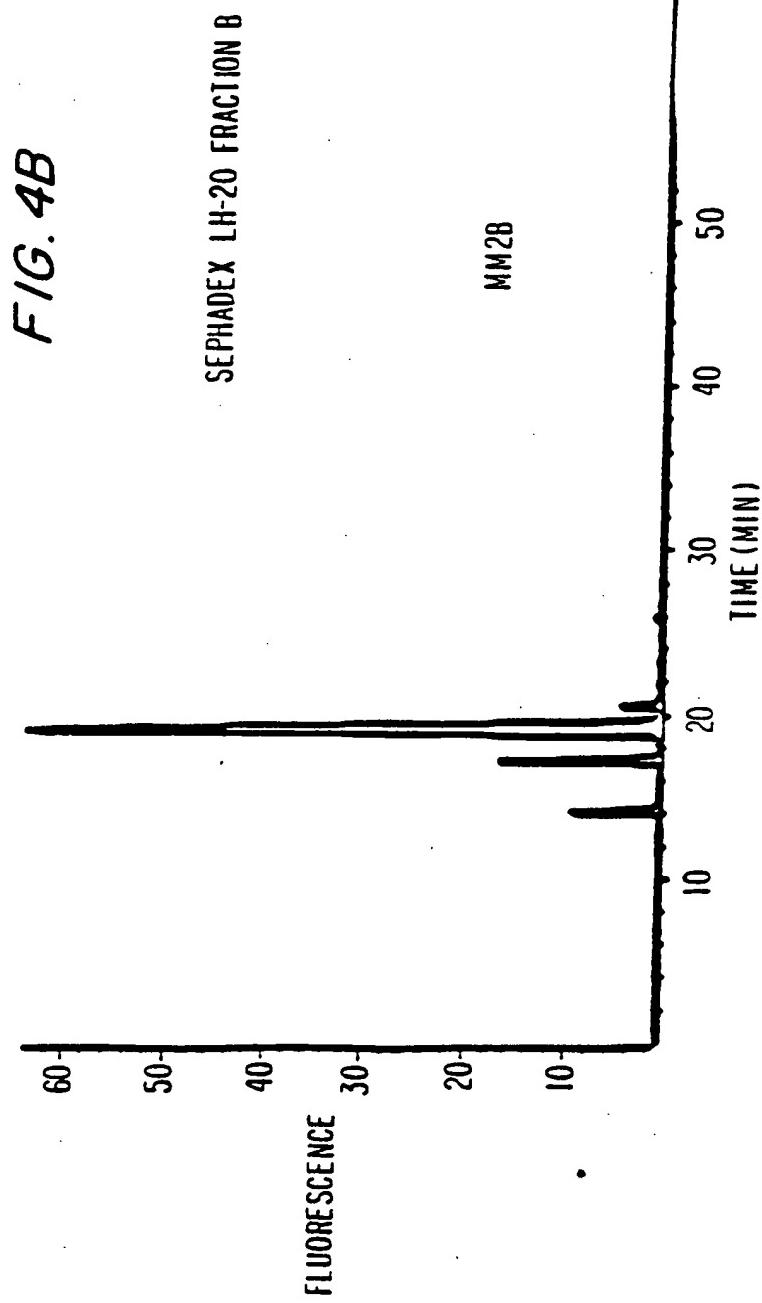
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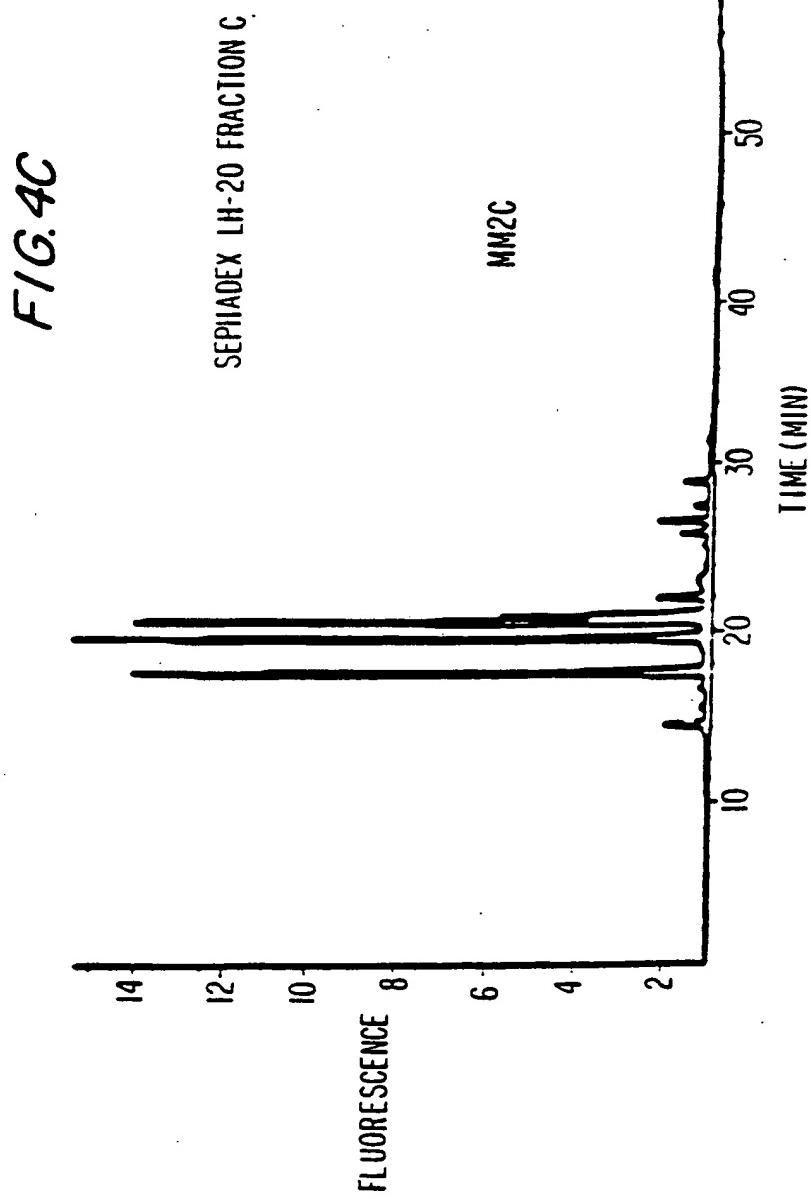
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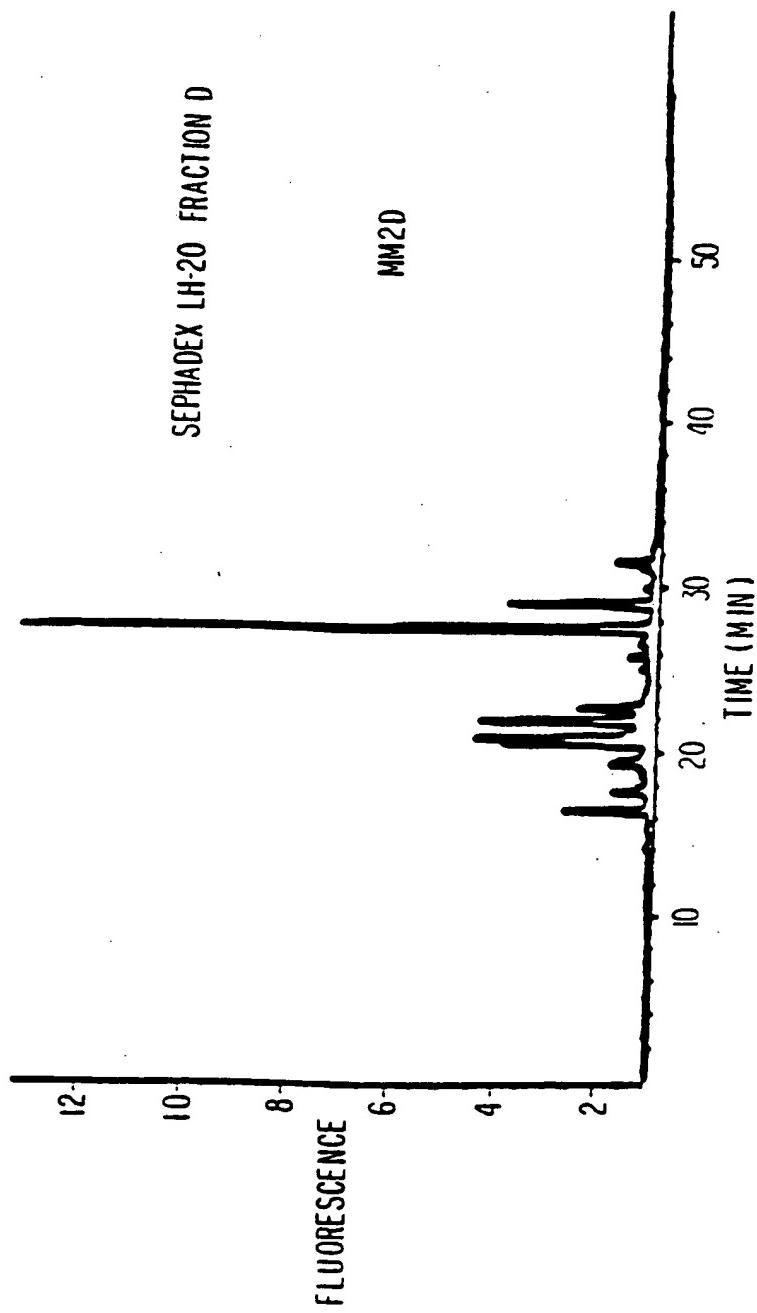
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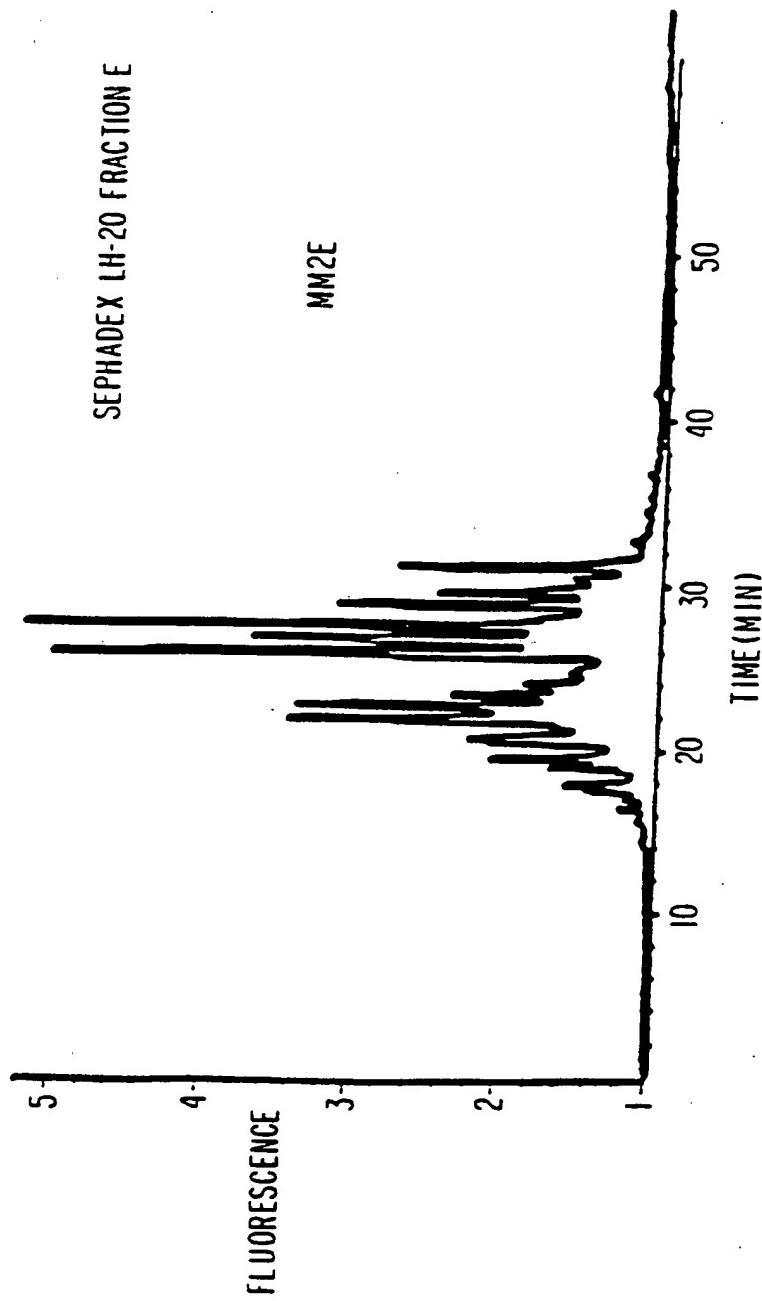
FIG. 4D



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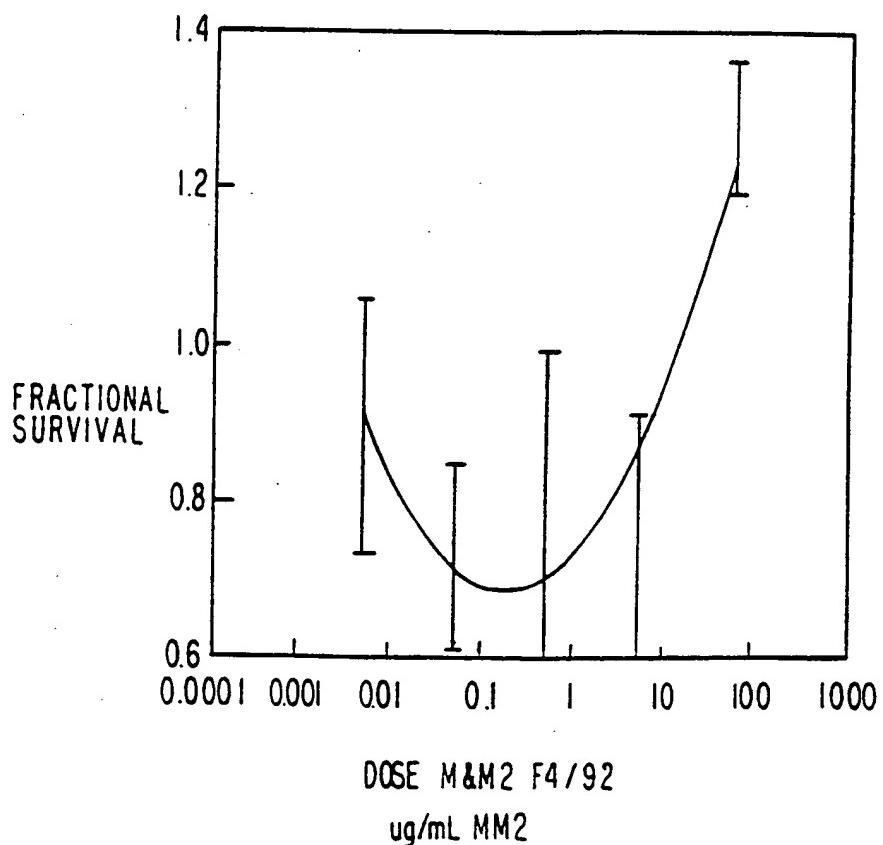
FIG. 4E



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FIG. 5



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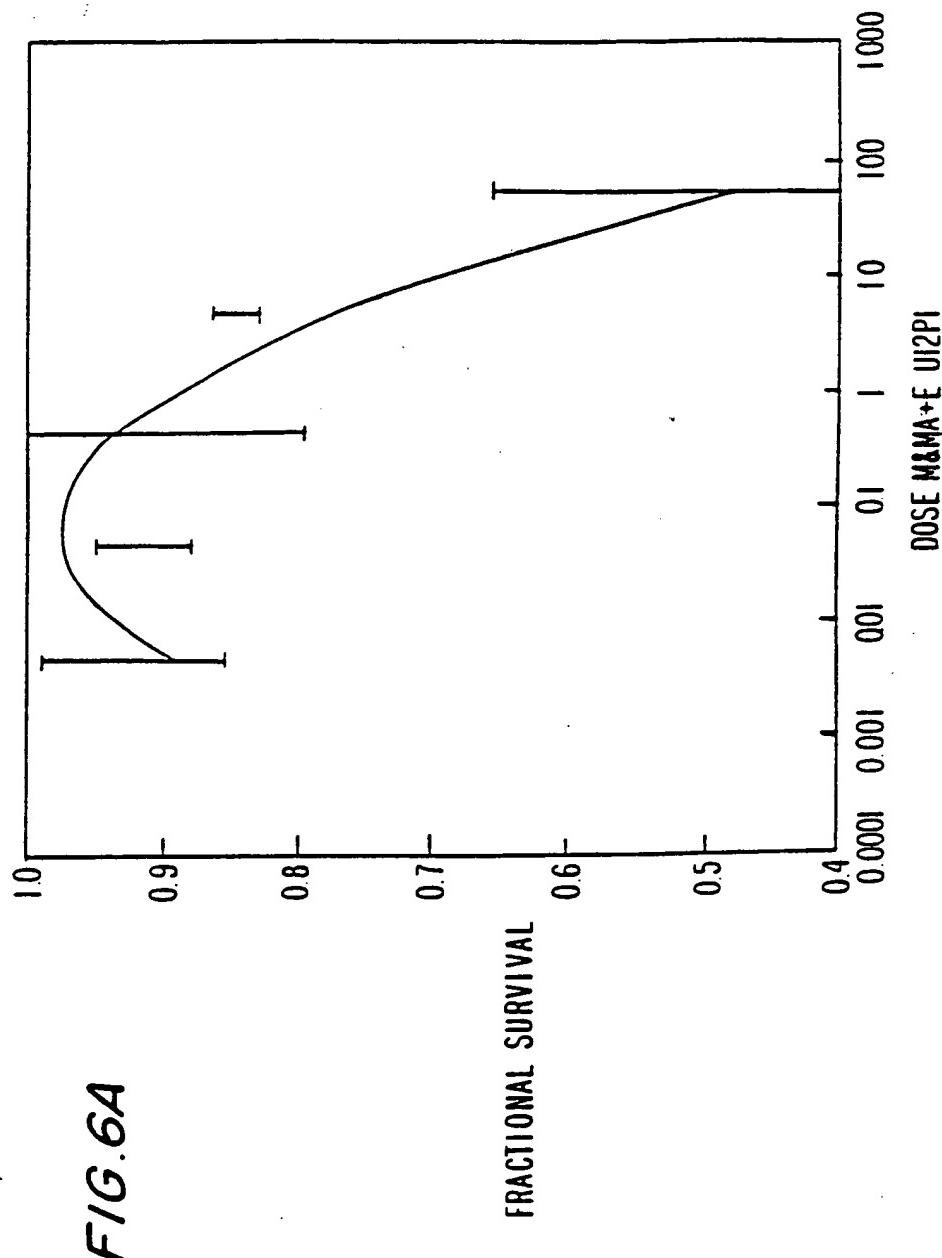


FIG. 6A

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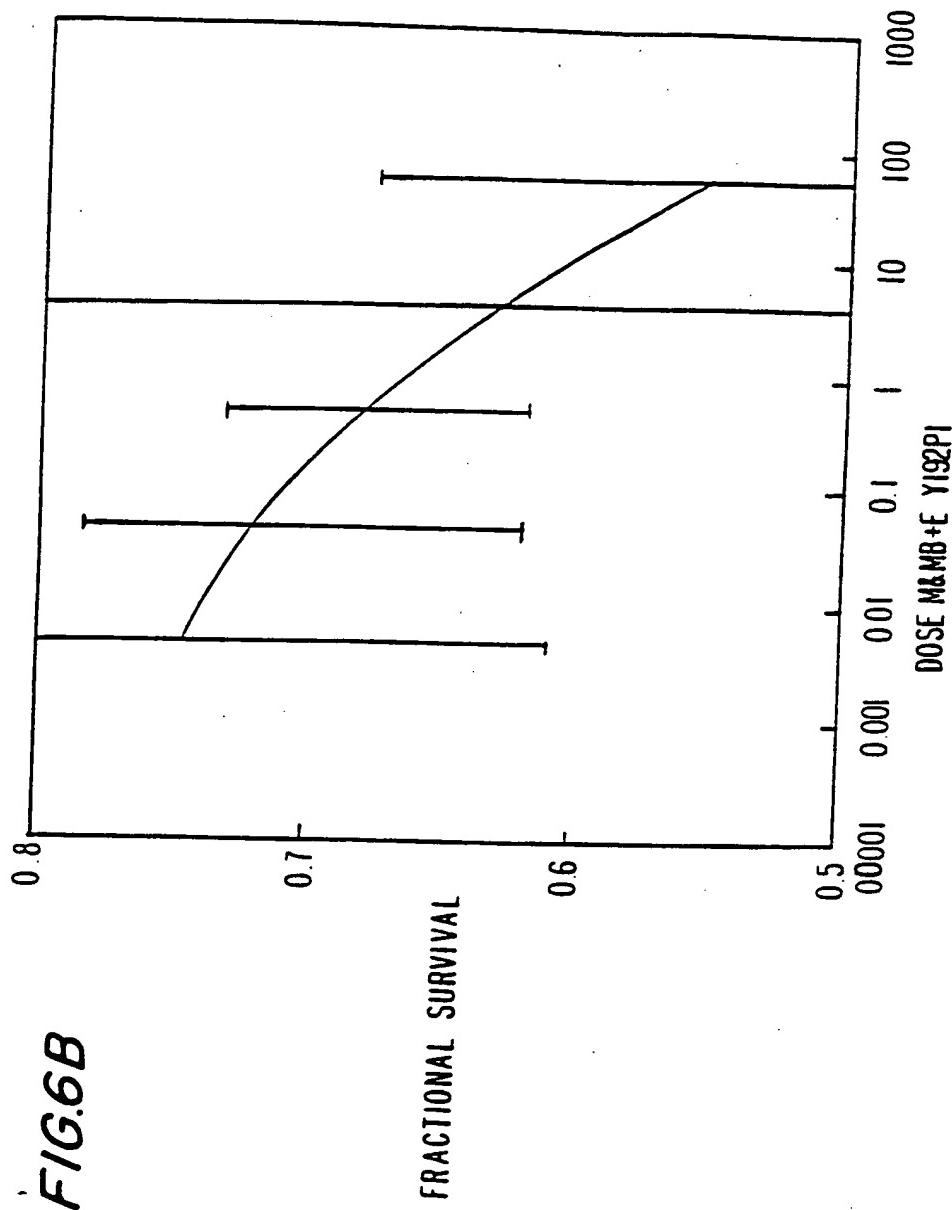


FIG. 6B

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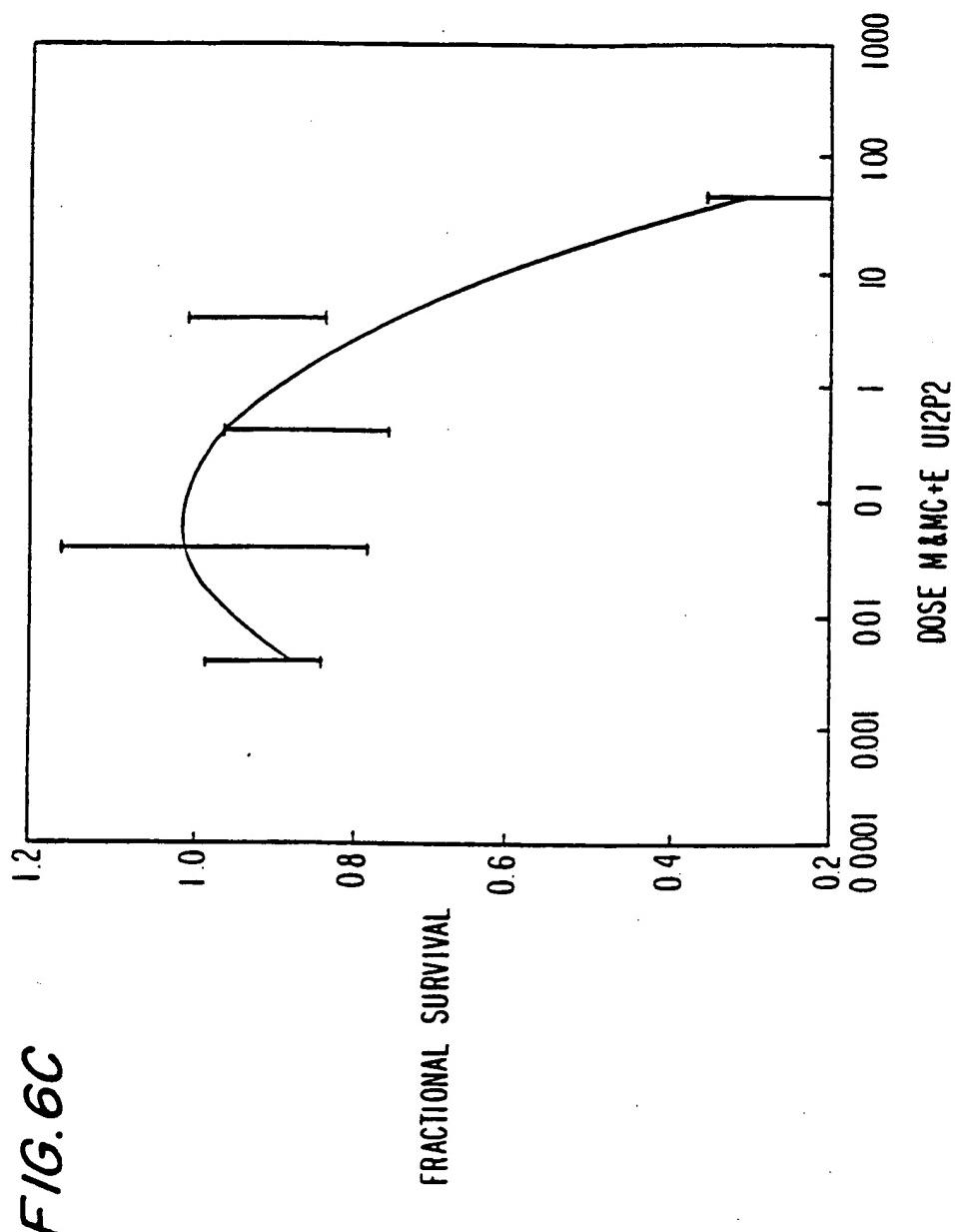


FIG. 6C

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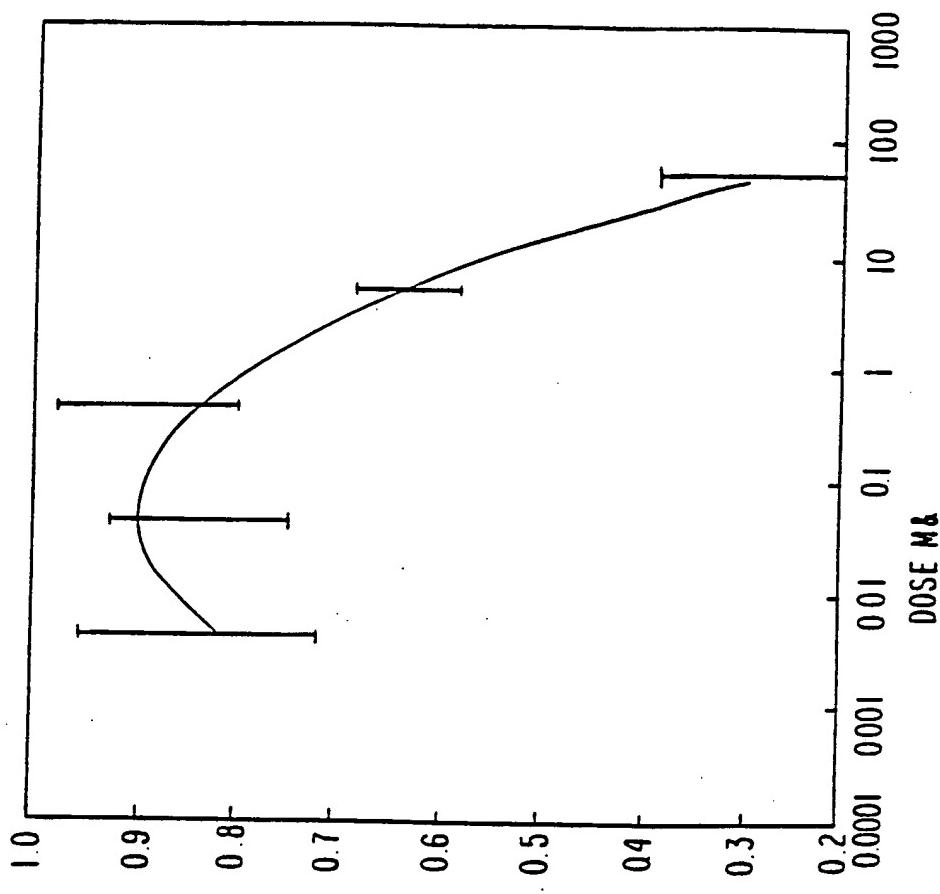


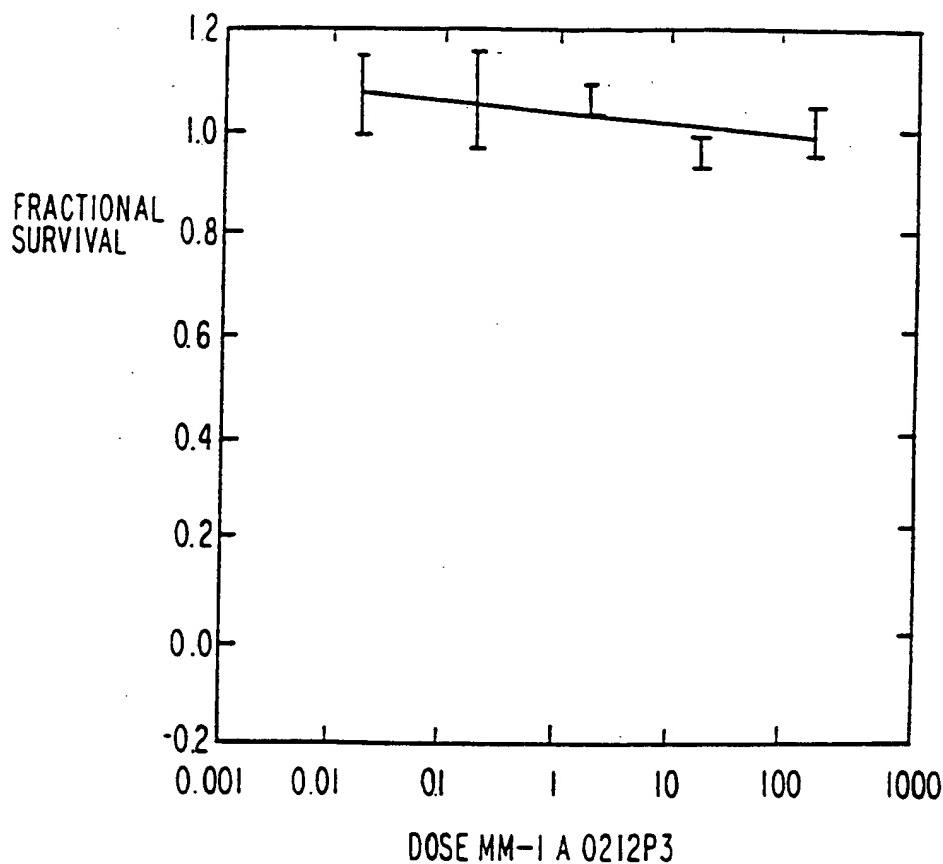
FIG. 6D

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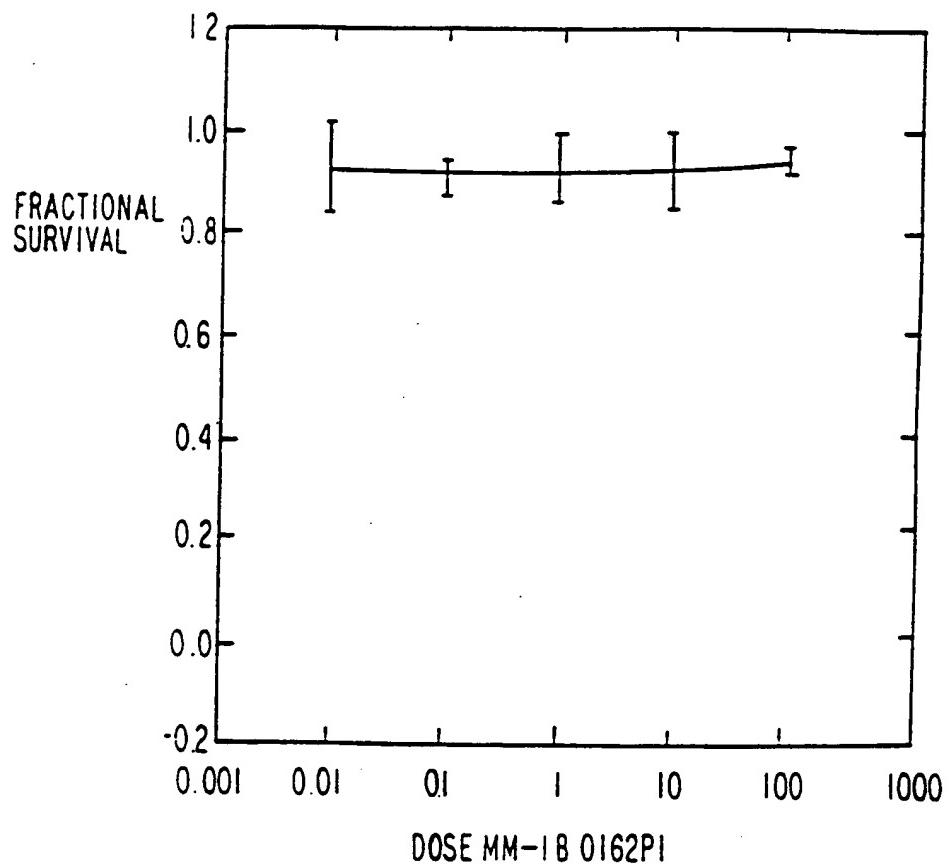
FIG. 7A



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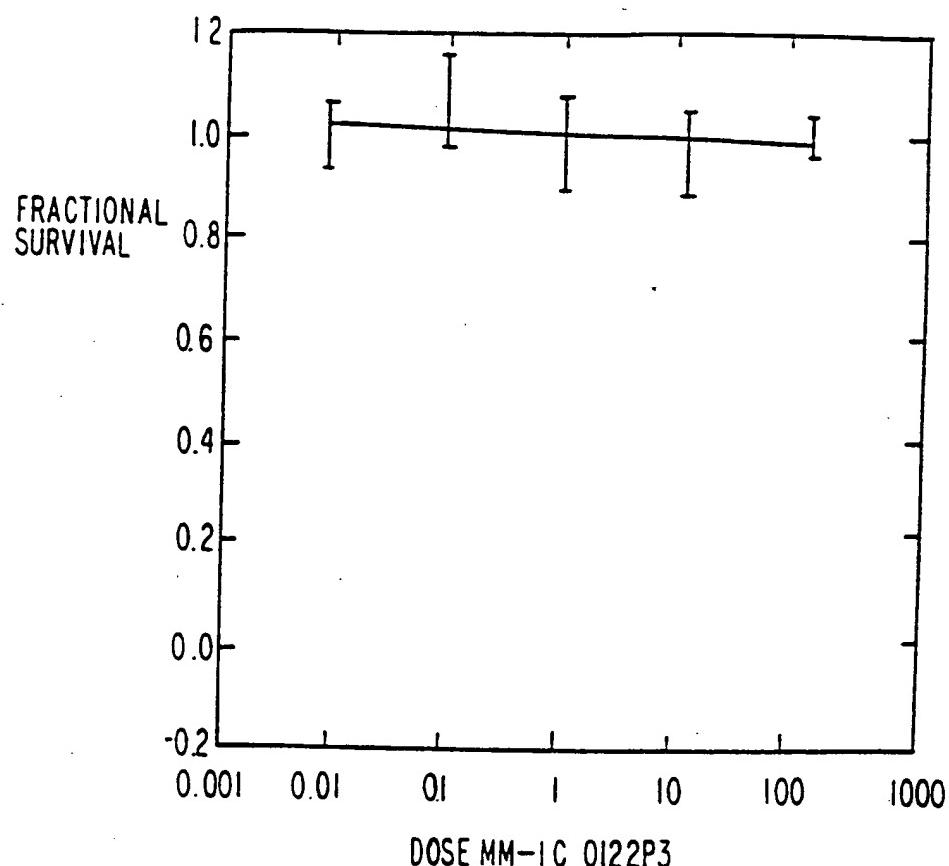
FIG. 7B



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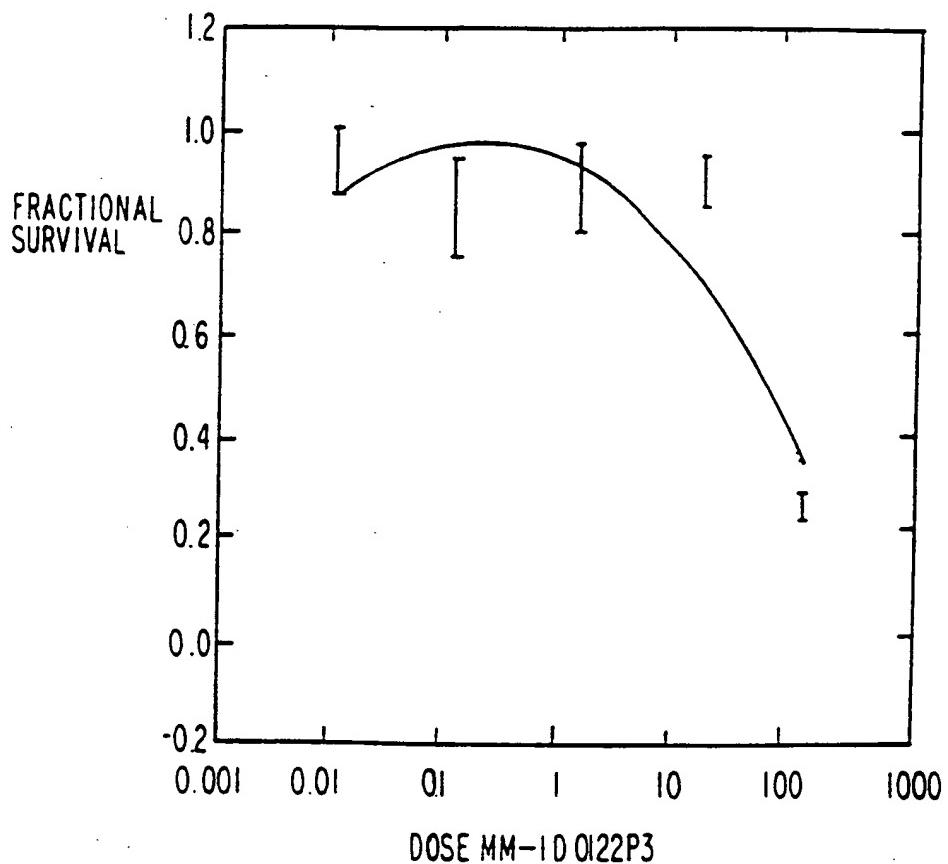
FIG. 7C



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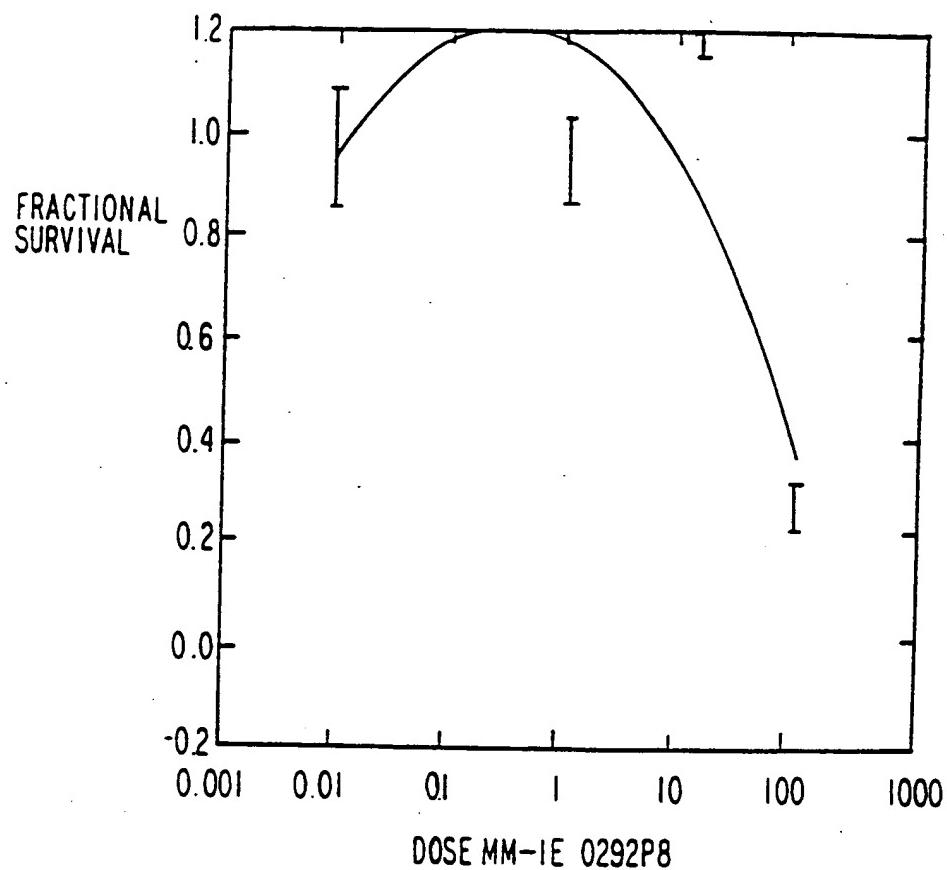
FIG. 7D



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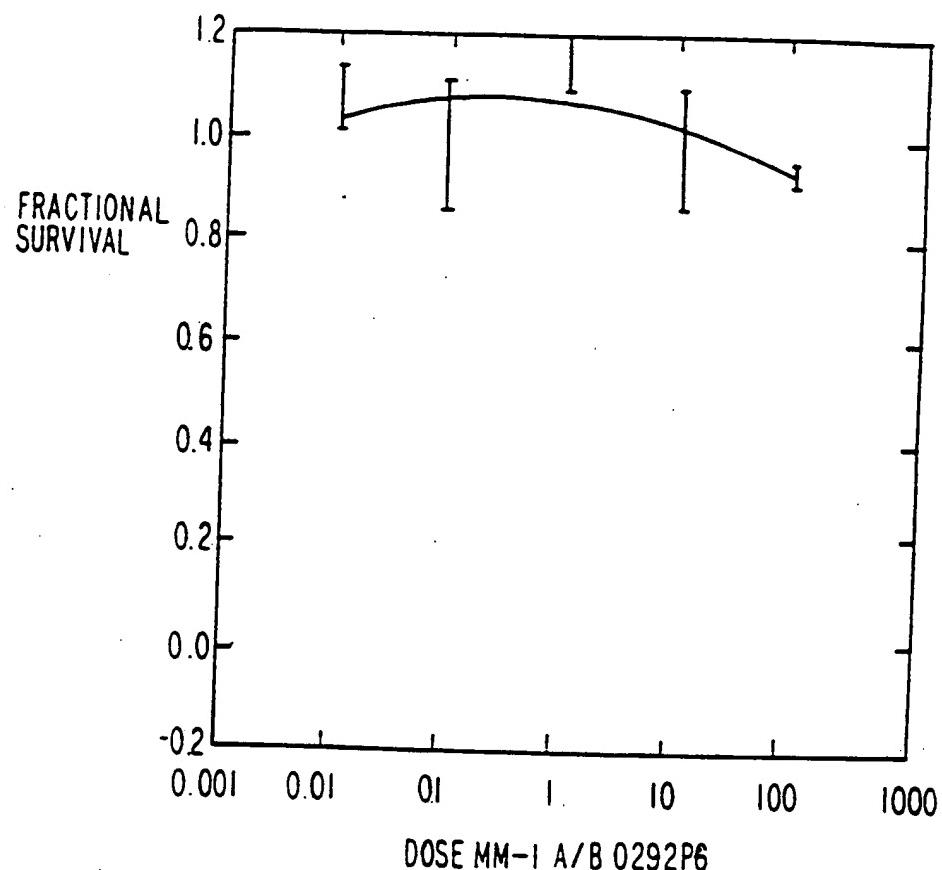
FIG. 7E



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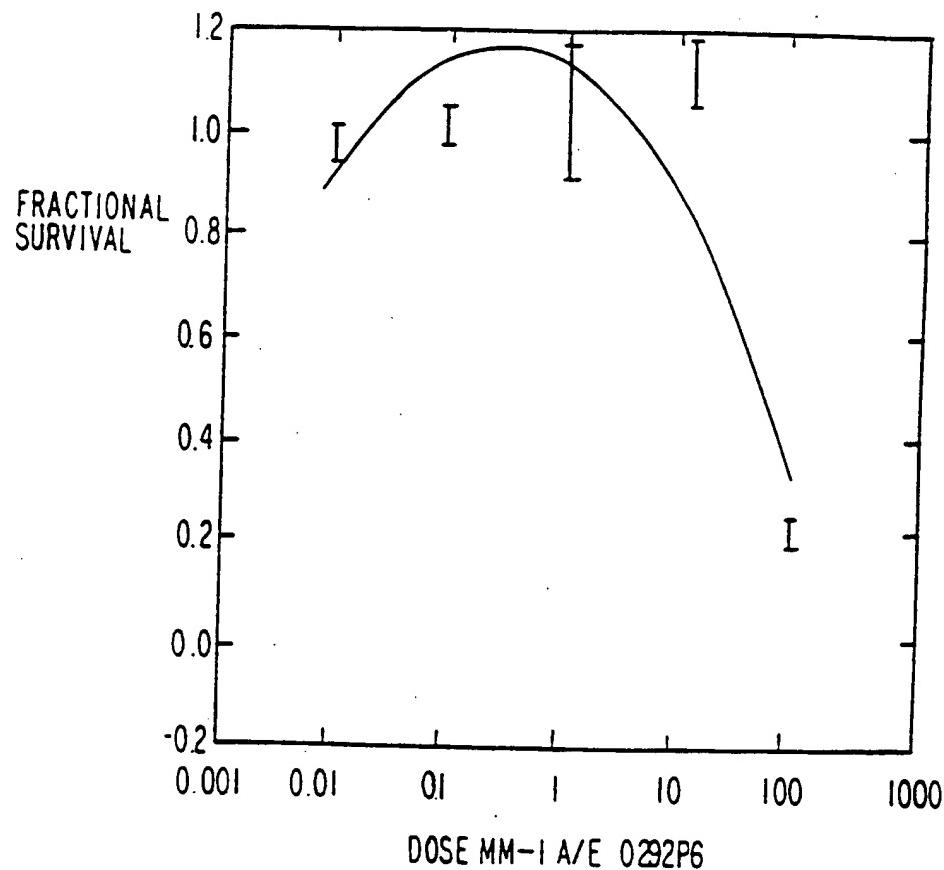
FIG. 7F



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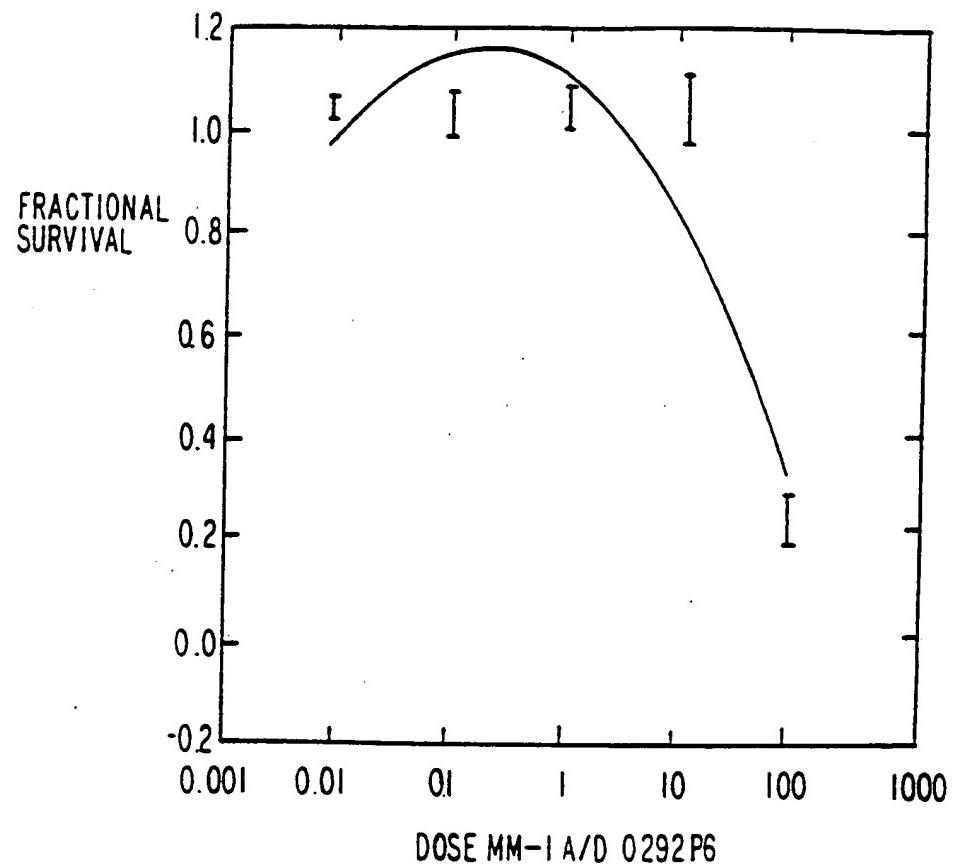
FIG. 7G



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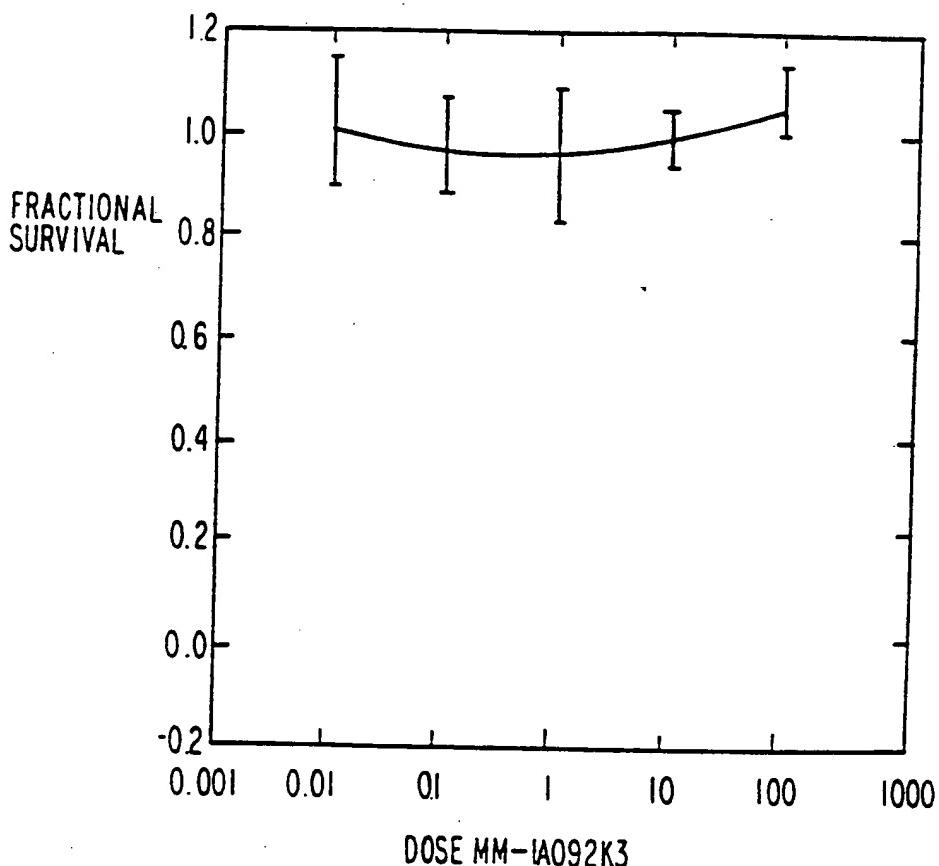
FIG. 7H



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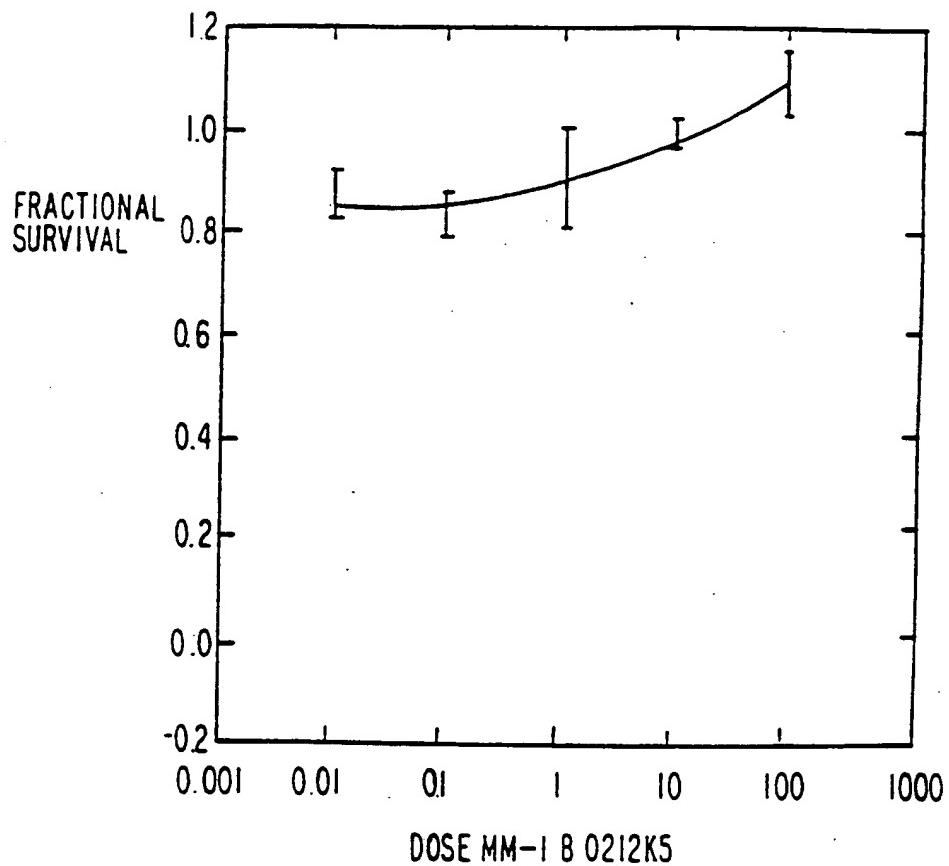
FIG.8A



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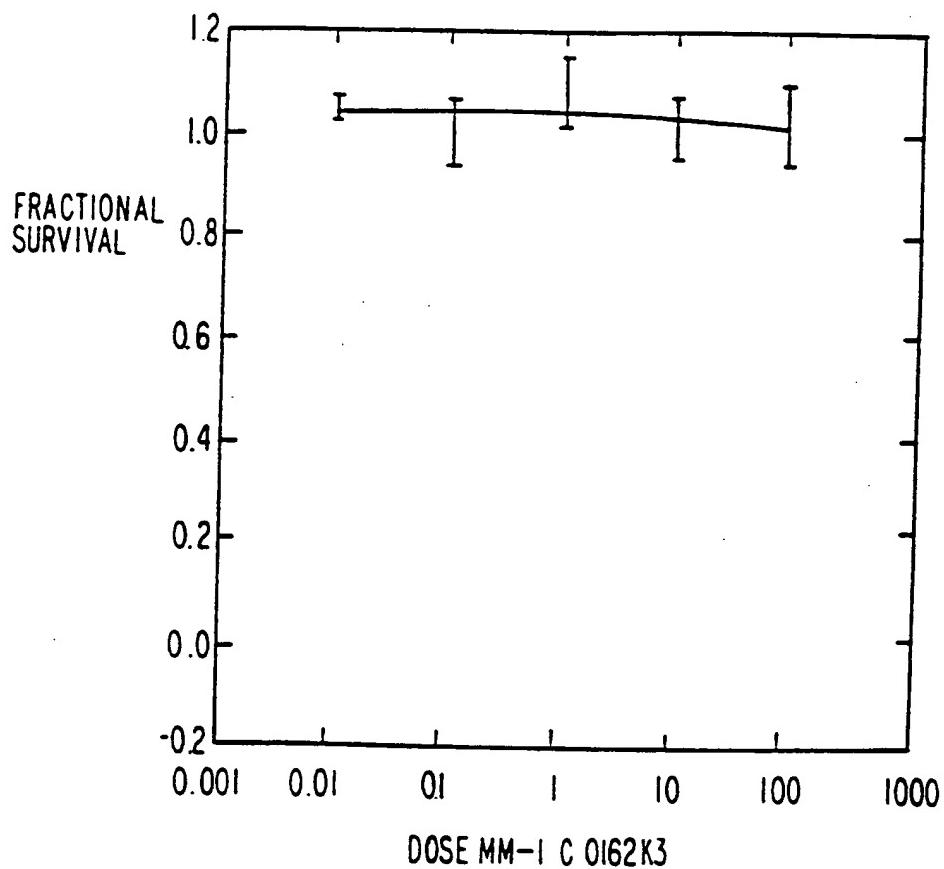
FIG. 8B



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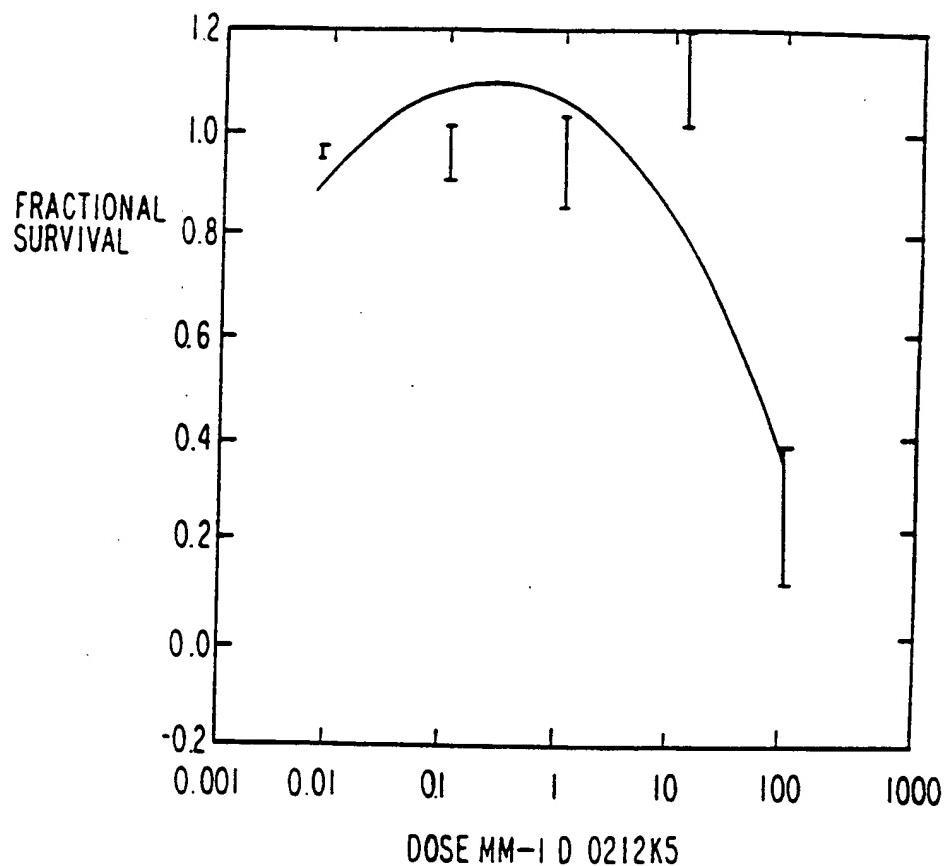
FIG. 8C



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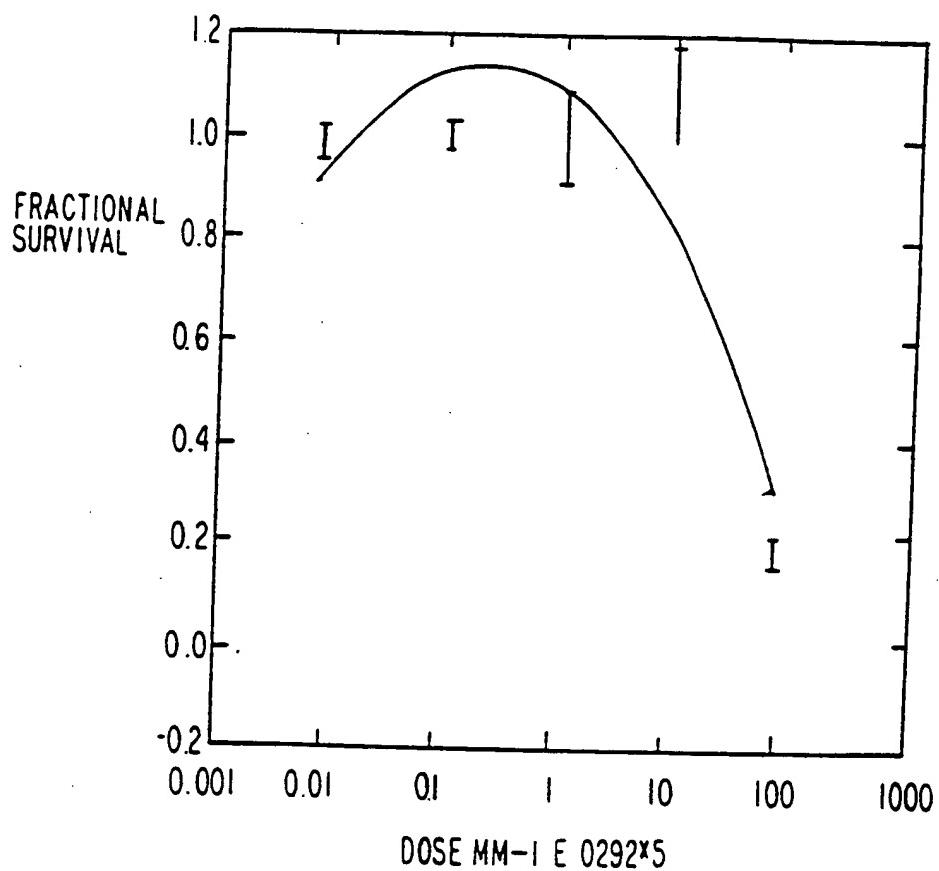
FIG. 8D



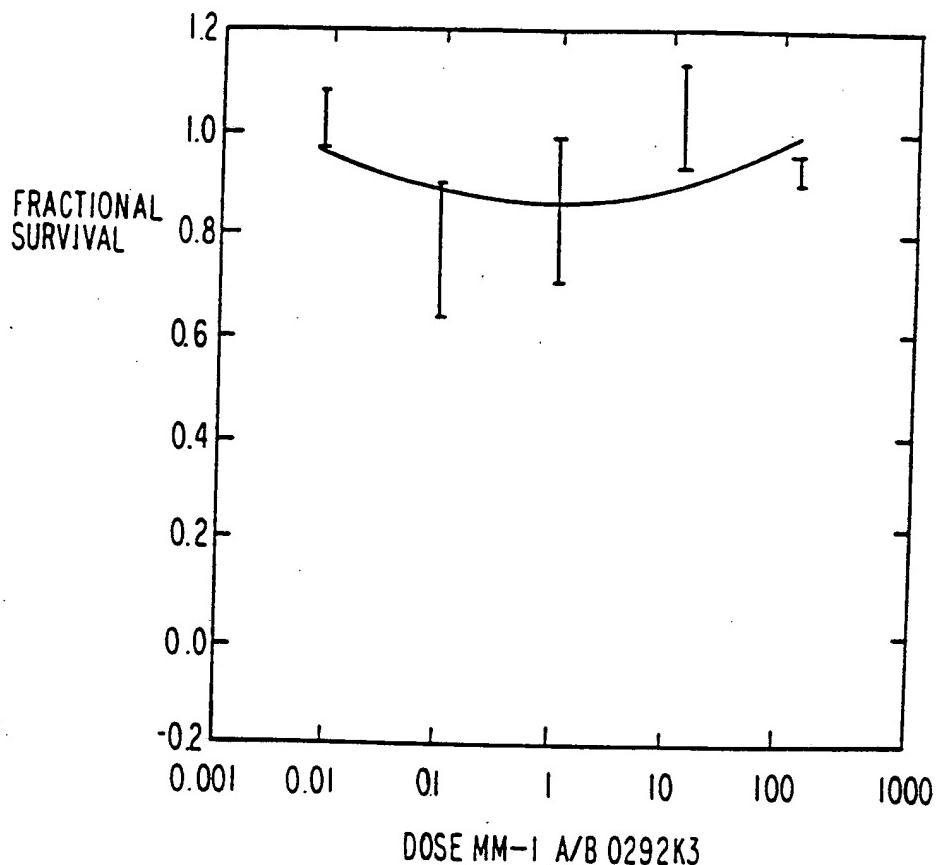
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FIG. 8E

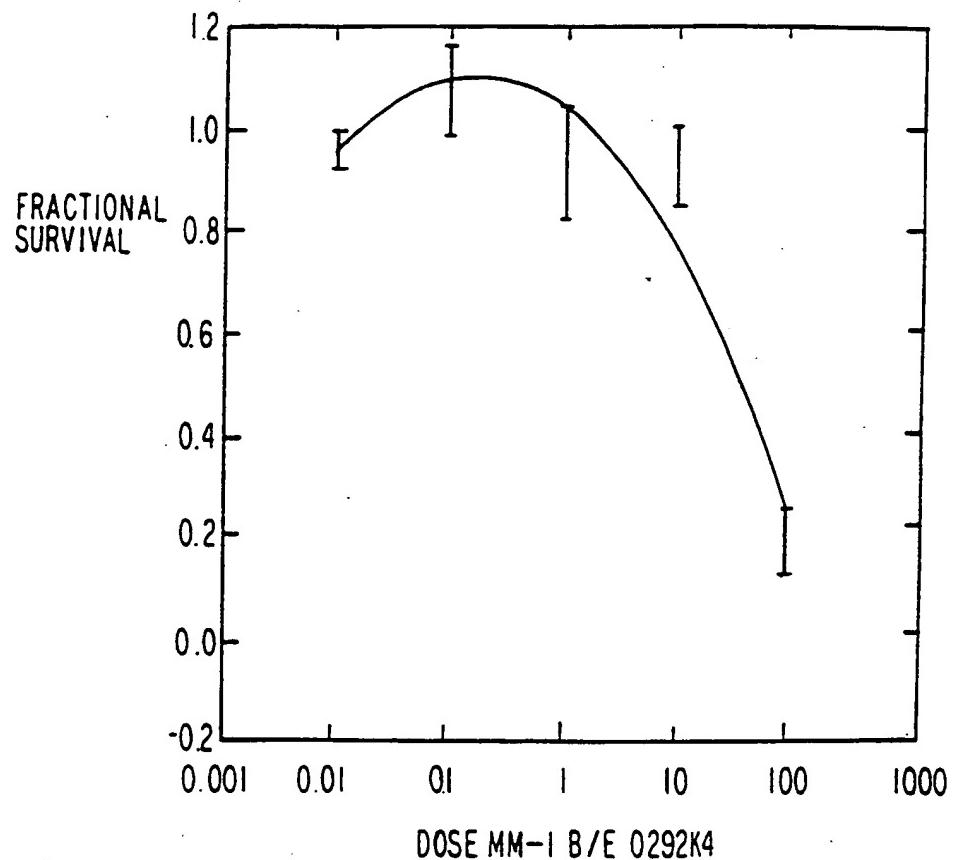


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FIG. 8F**SUBSTITUTE SHEET (RULE 26)**

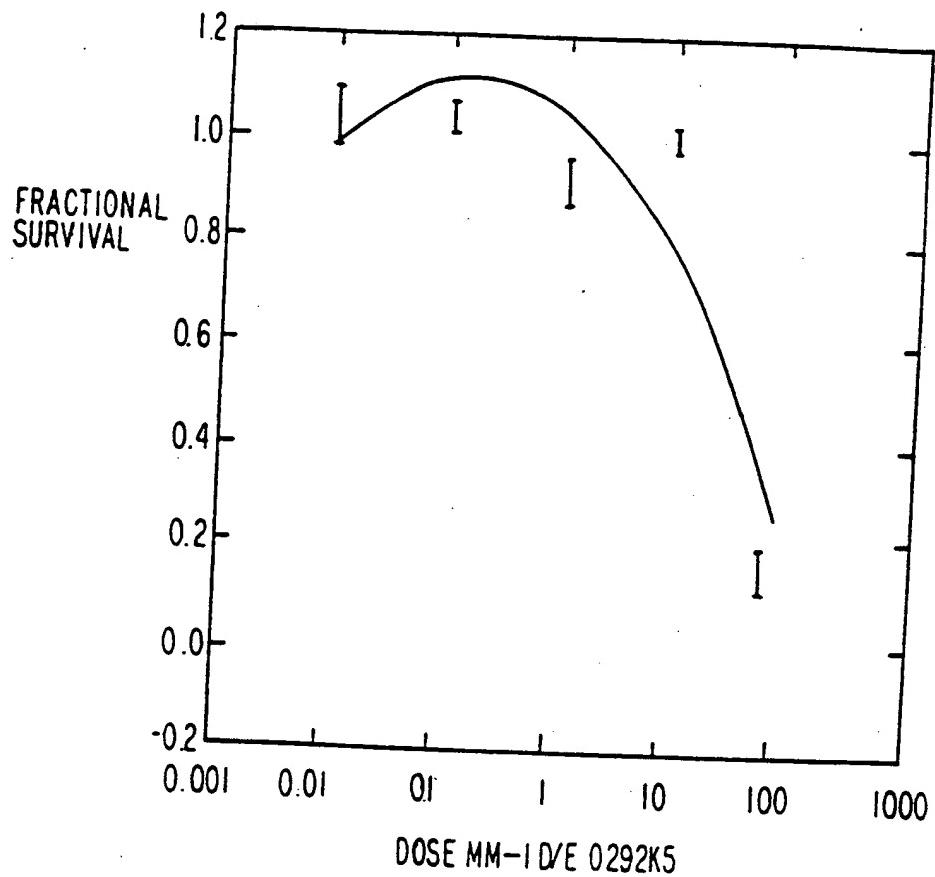
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FIG. 8G



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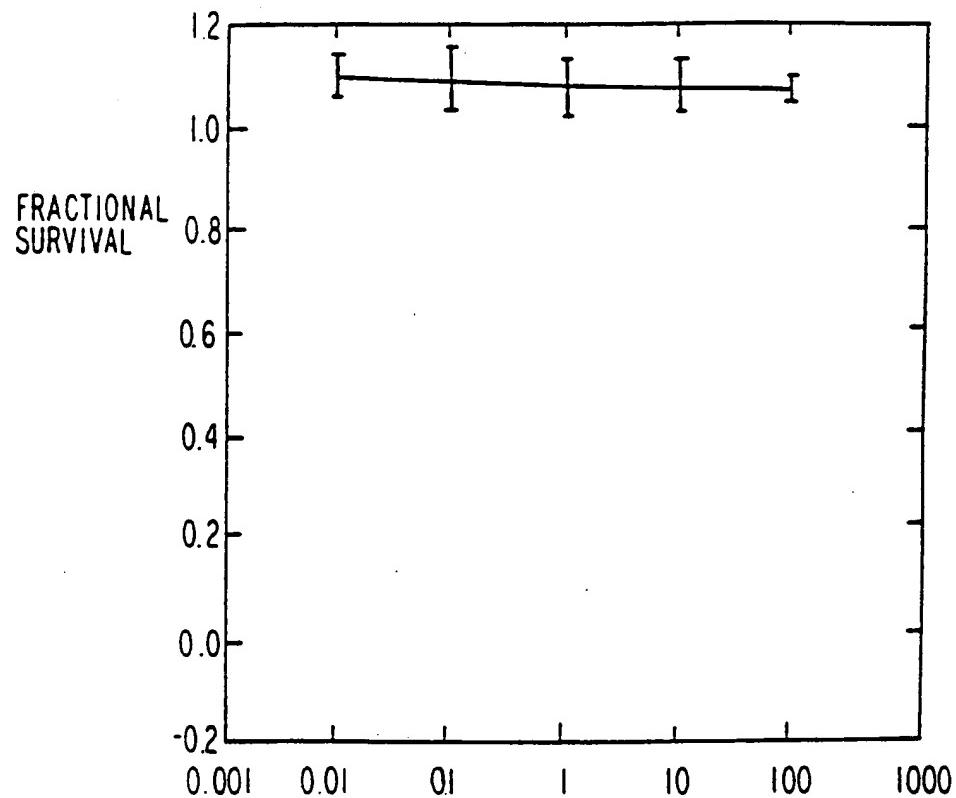
FIG. 8H



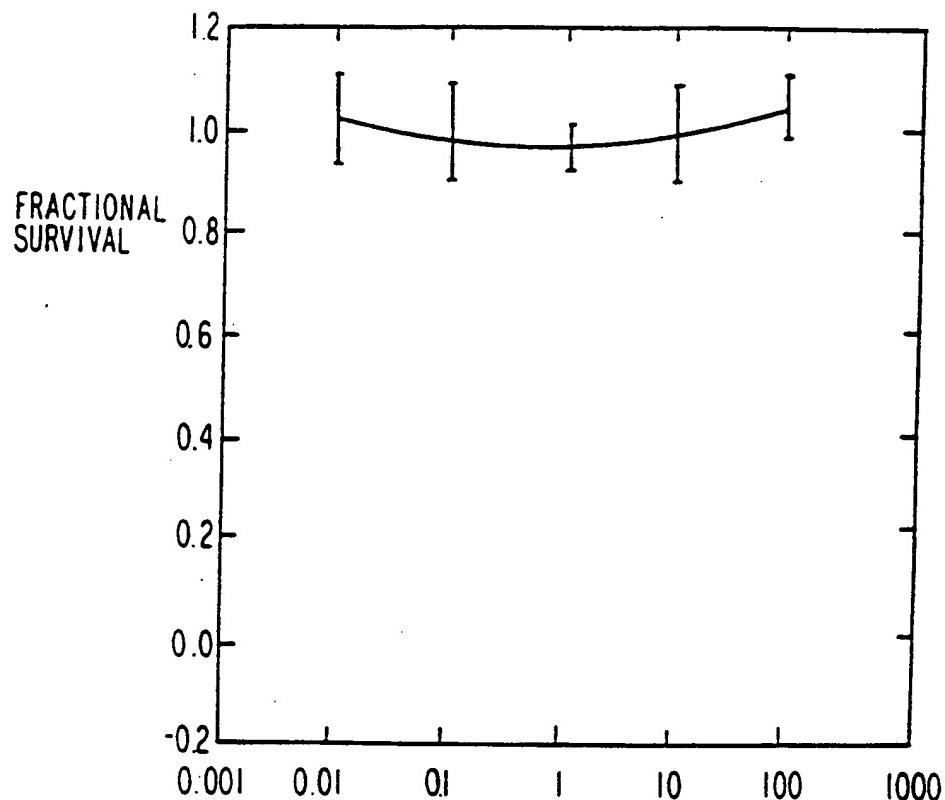
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FIG. 9A

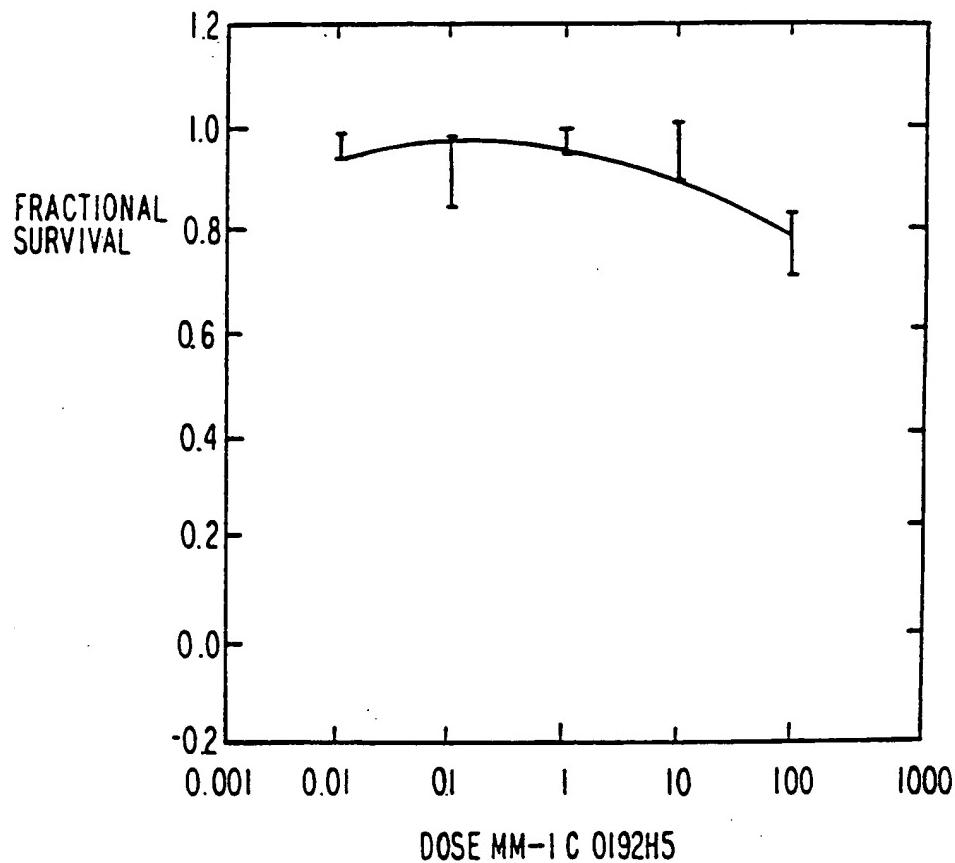


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FIG. 9B**SUBSTITUTE SHEET (RULE 26)**

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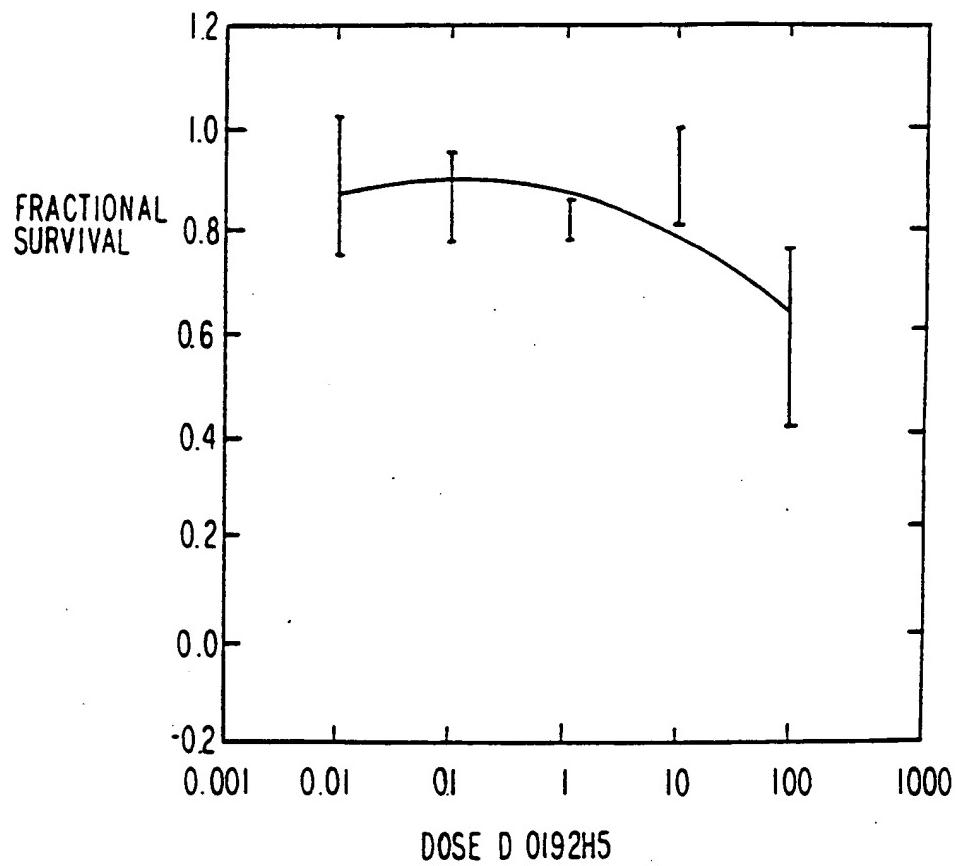
FIG. 9C



SUBSTITUTE SHEET (RULE 26)

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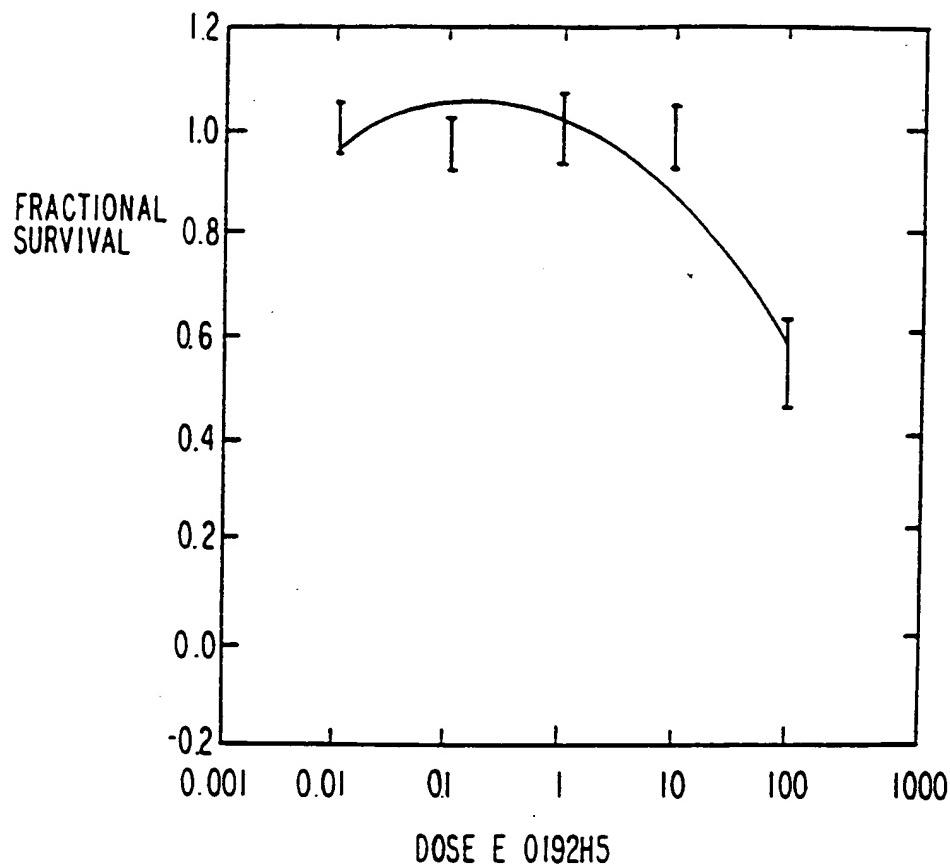
FIG. 9D



SUBSTITUTE SHEET (RULE 26)

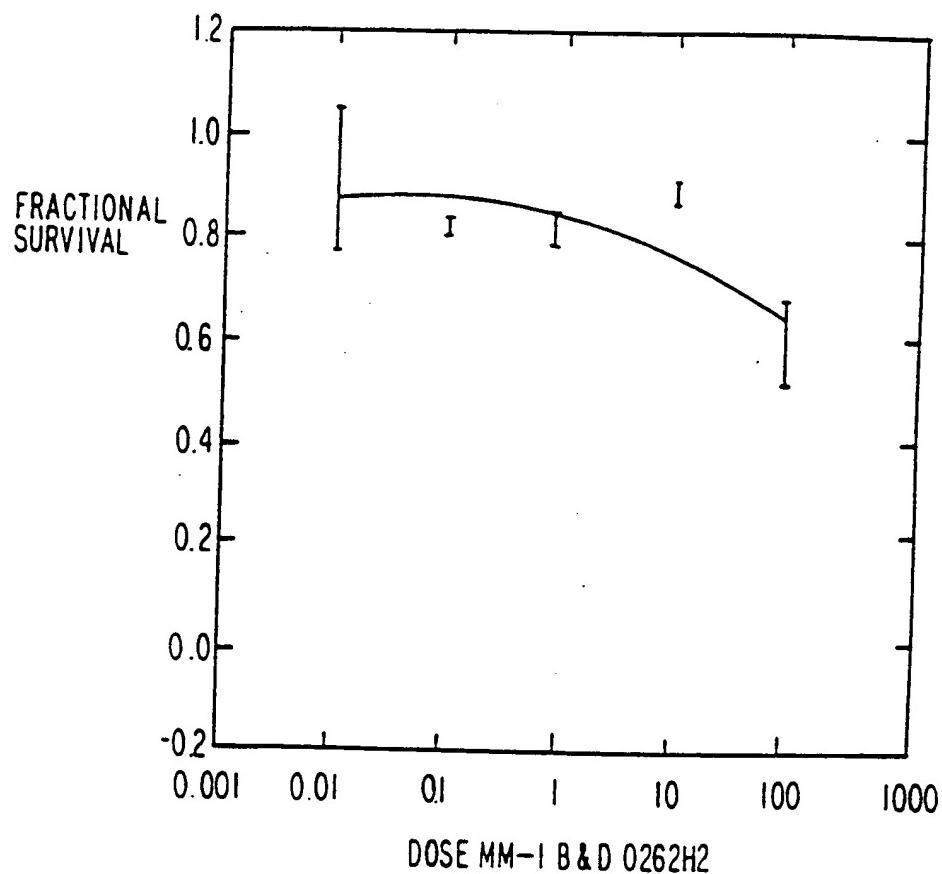
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FIG. 9E



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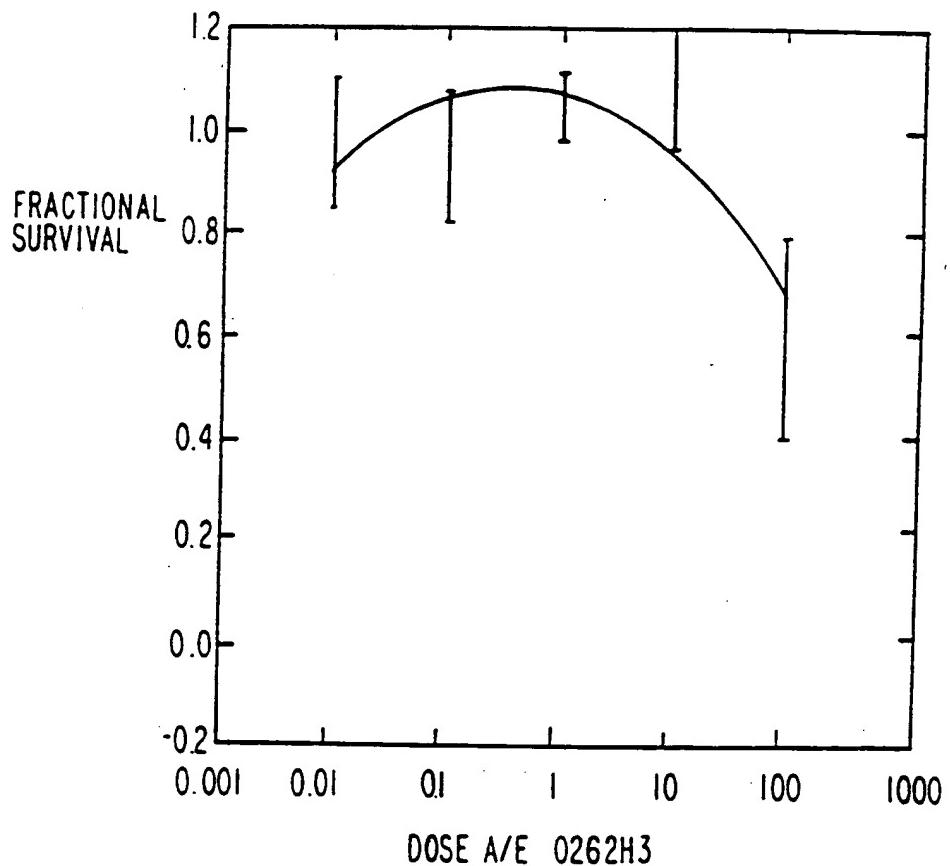
FIG. 9F



SUBSTITUTE SHEET (RULE 26)

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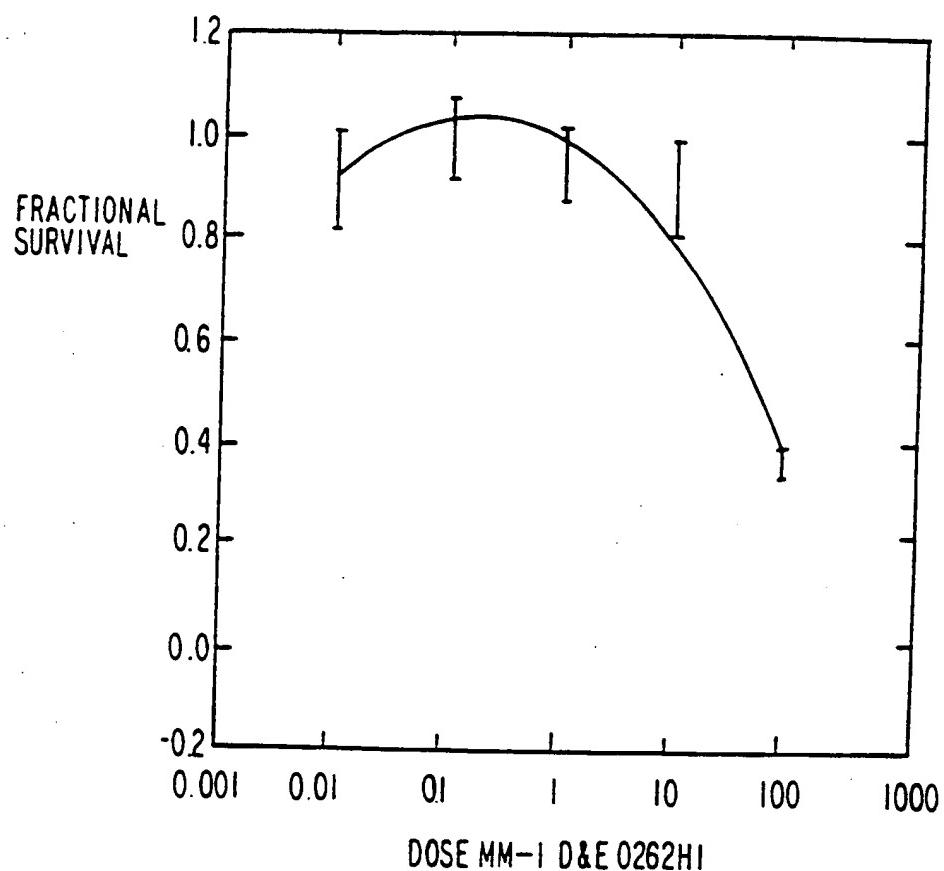
FIG. 9G



SUBSTITUTE SHEET (RULE 26)

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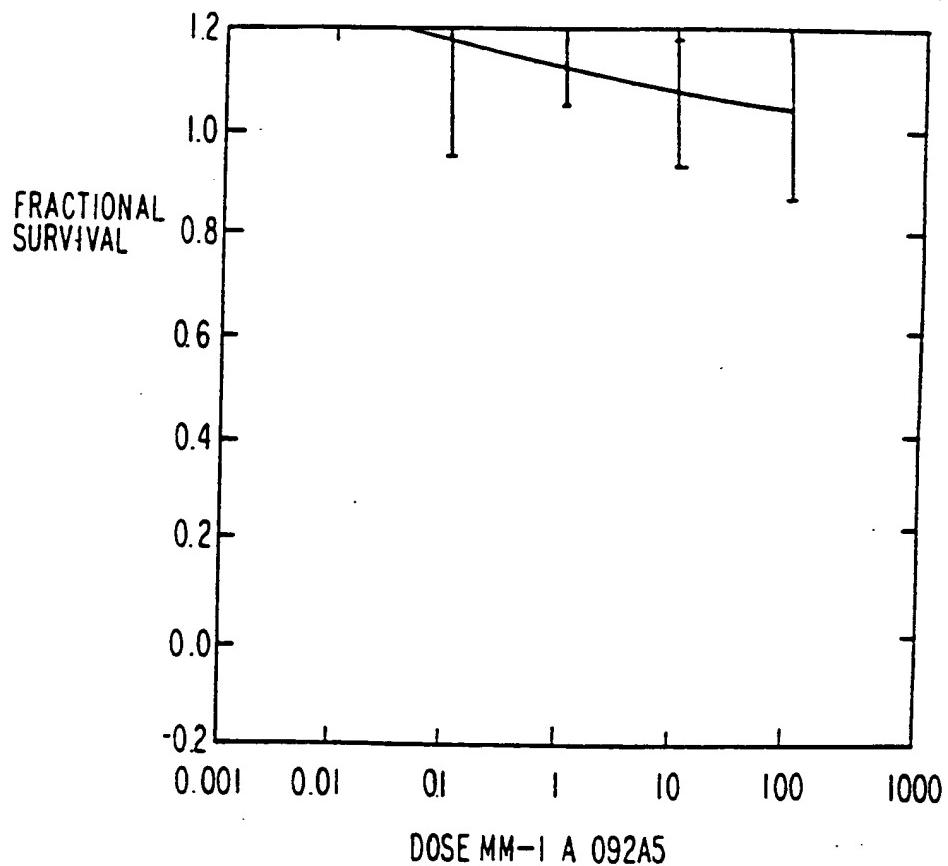
FIG. 9H



SUBSTITUTE SHEET (RULE 26)

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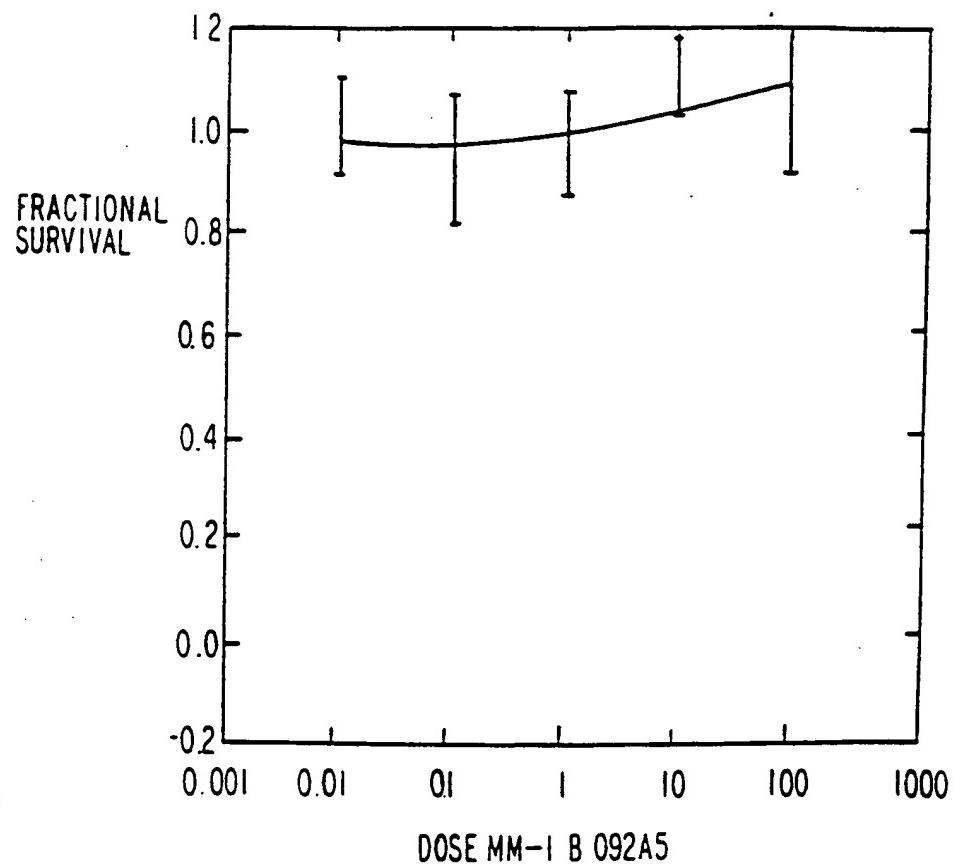
FIG. 10A



SUBSTITUTE SHEET (RULE 26)

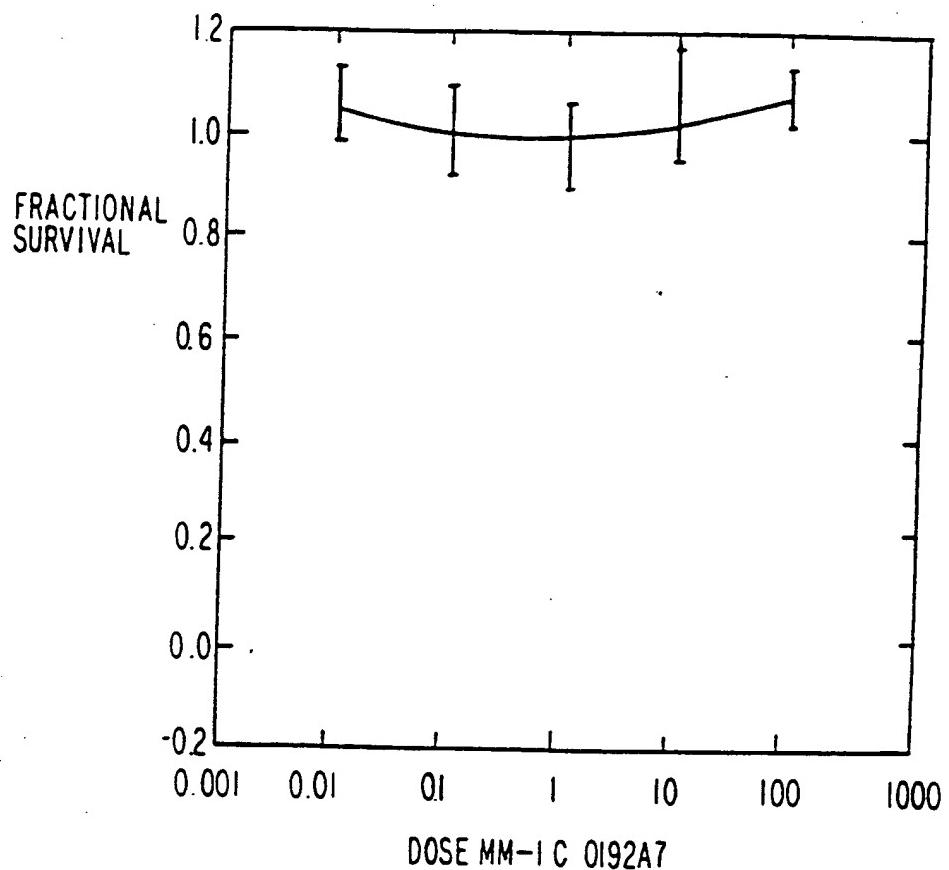
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FIG. 10B



SUBSTITUTE SHEET (RULE 26)

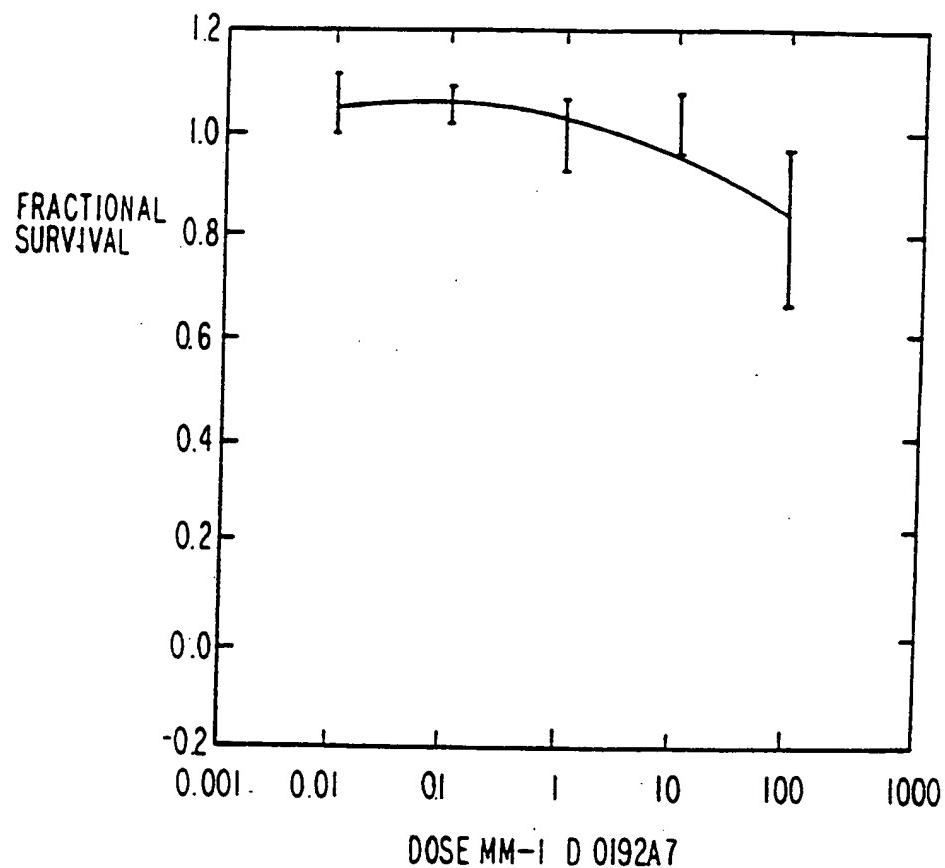
FIG. 10C



SUBSTITUTE SHEET (RULE 26)

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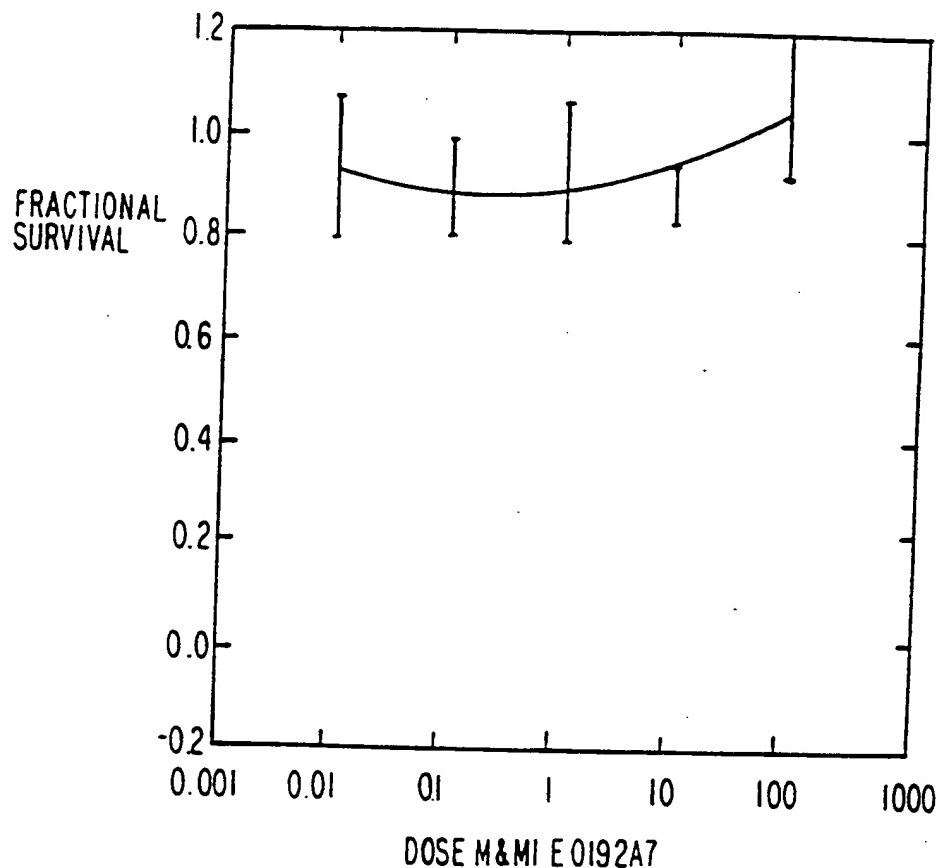
FIG. 1OD



SUBSTITUTE SHEET (RULE 26)

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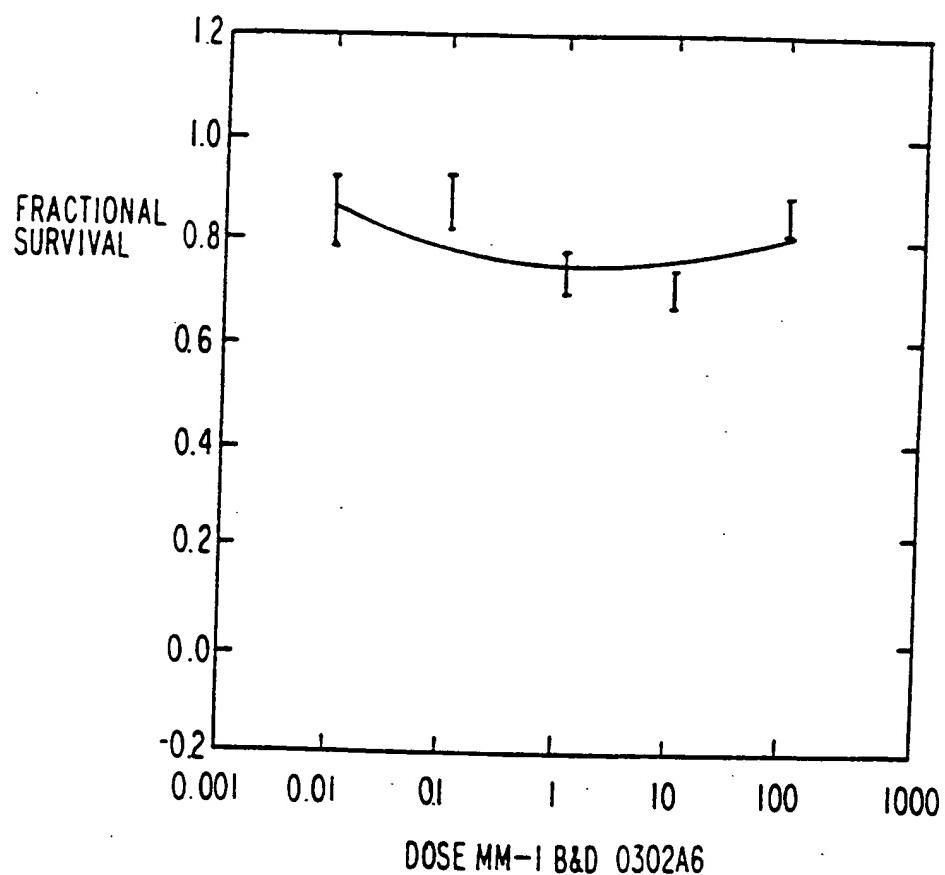
FIG. 10E



SUBSTITUTE SHEET (RULE 26)

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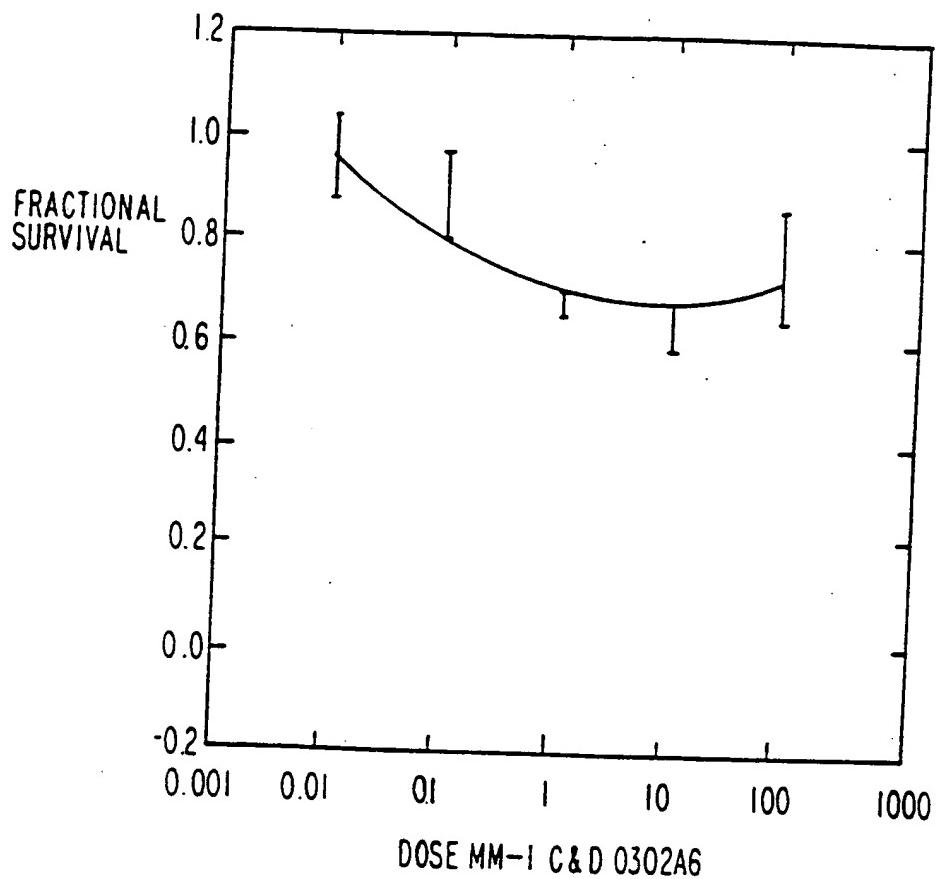
FIG. 10F



SUBSTITUTE SHEET (RULE 26)

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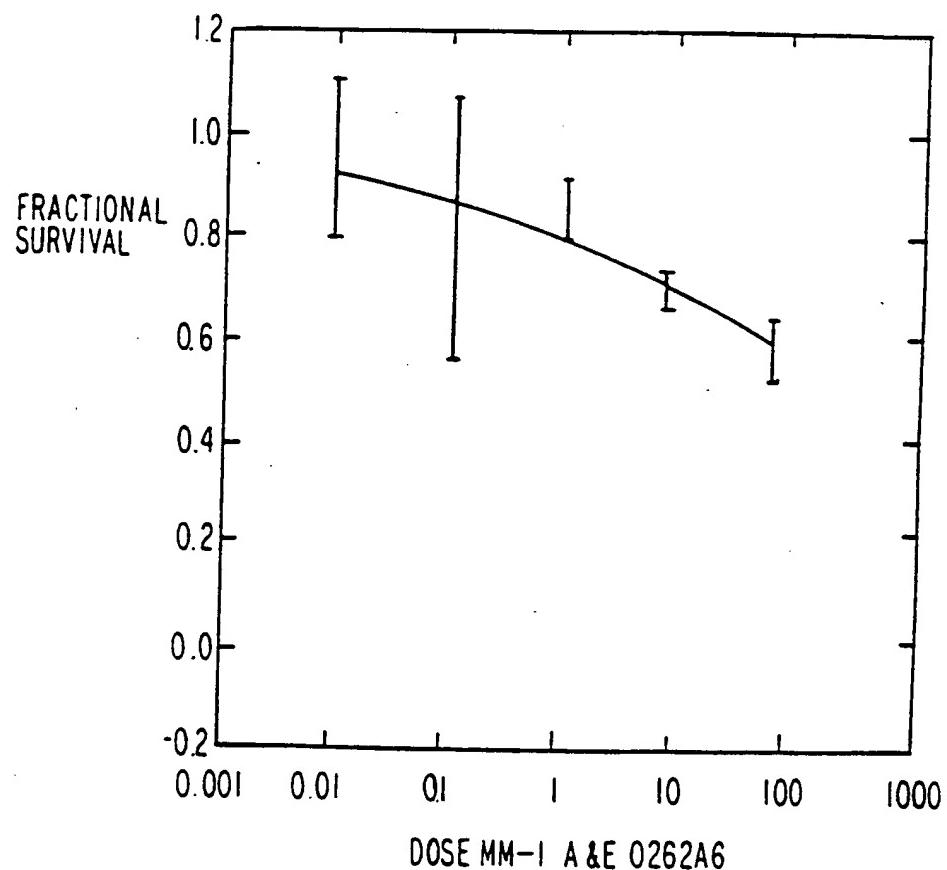
FIG.10G



SUBSTITUTE SHEET (RULE 26)

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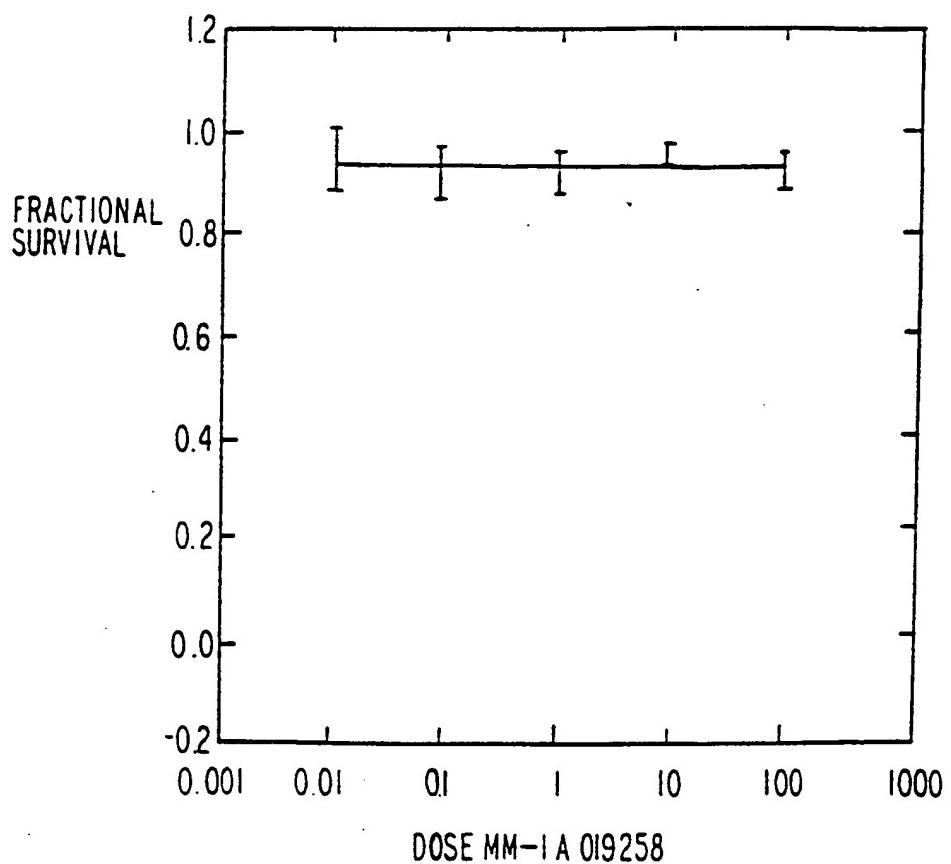
FIG. 10H



SUBSTITUTE SHEET (RULE 26)

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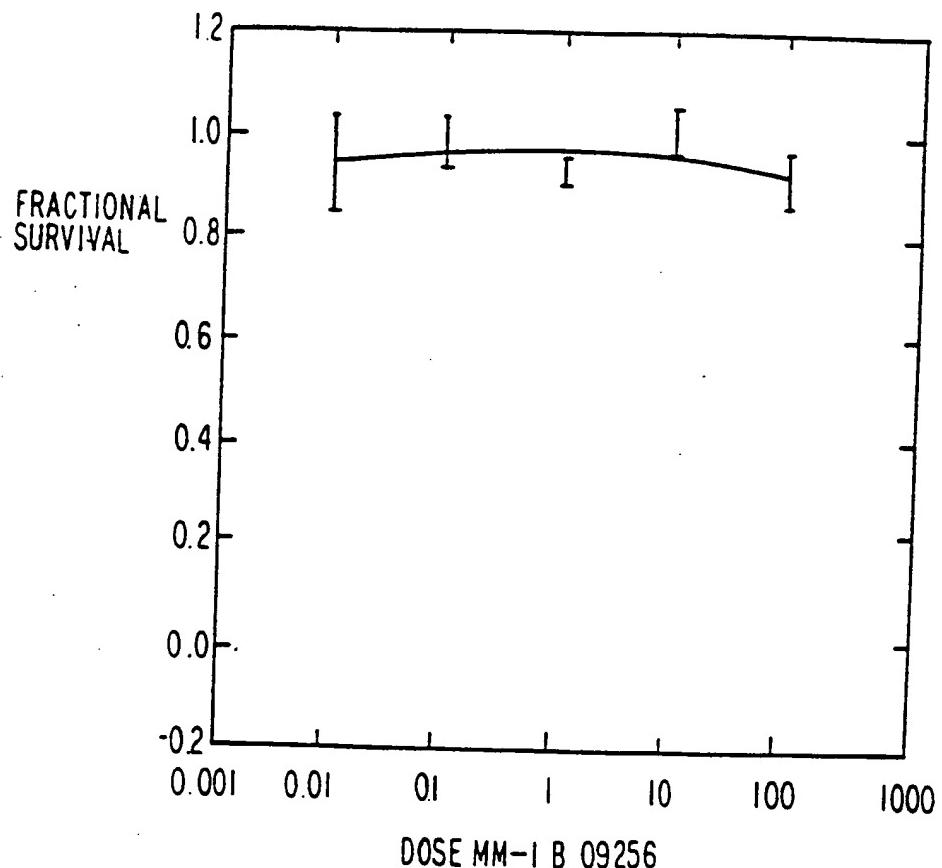
FIG. 11A



SUBSTITUTE SHEET (RULE 26)

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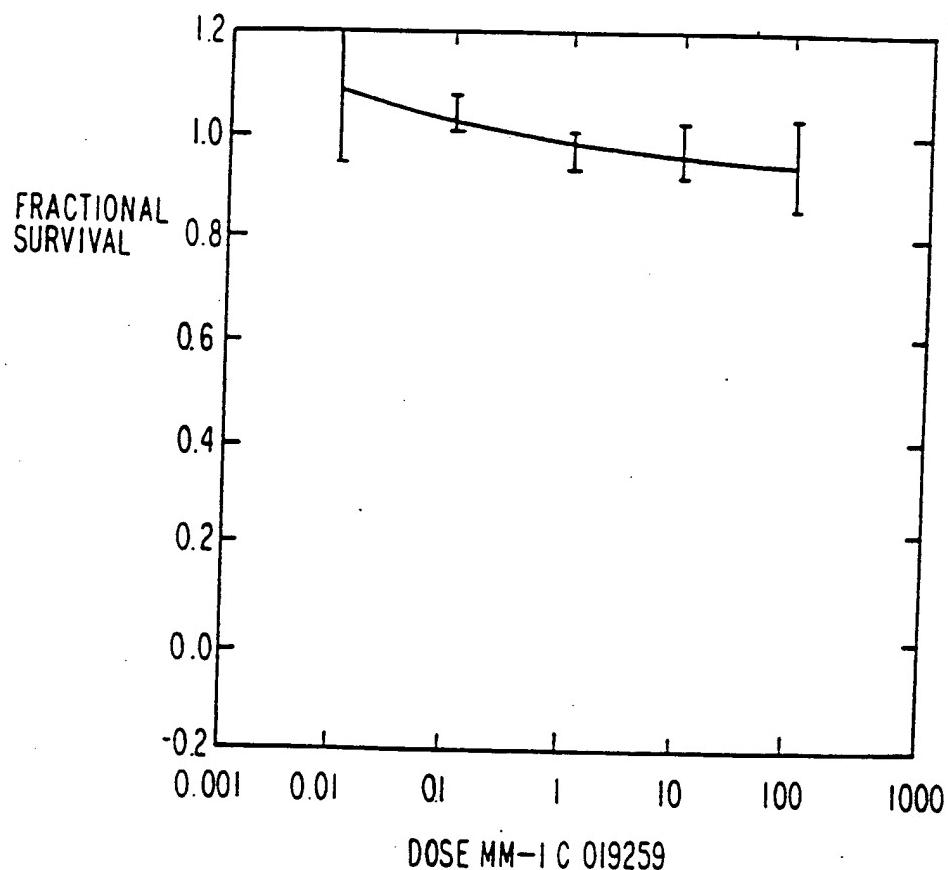
FIG. 11B



SUBSTITUTE SHEET (RULE 26)

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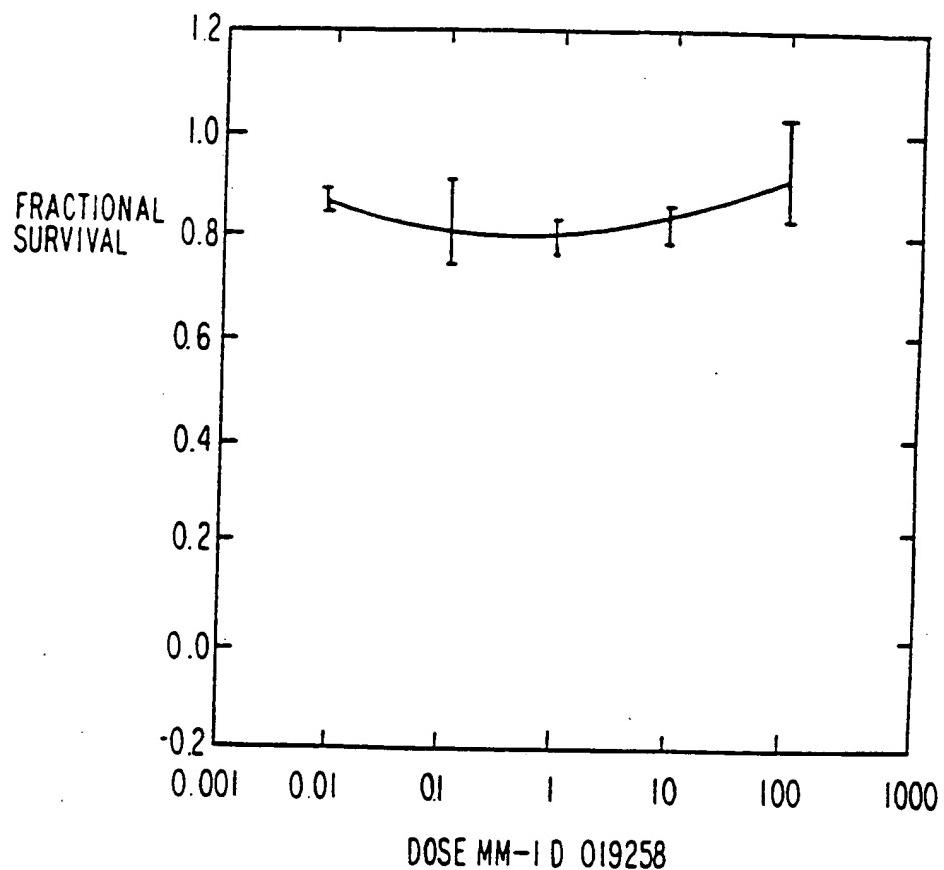
FIG. 11C



SUBSTITUTE SHEET (RULE 26)

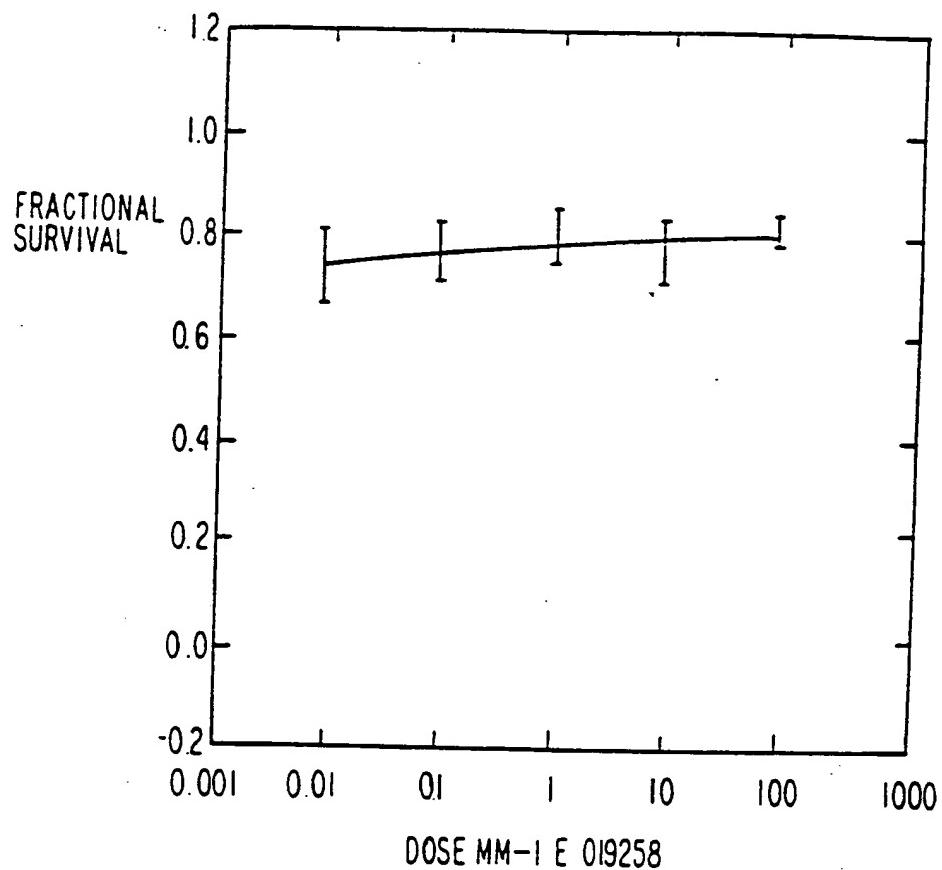
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FIG. 11D



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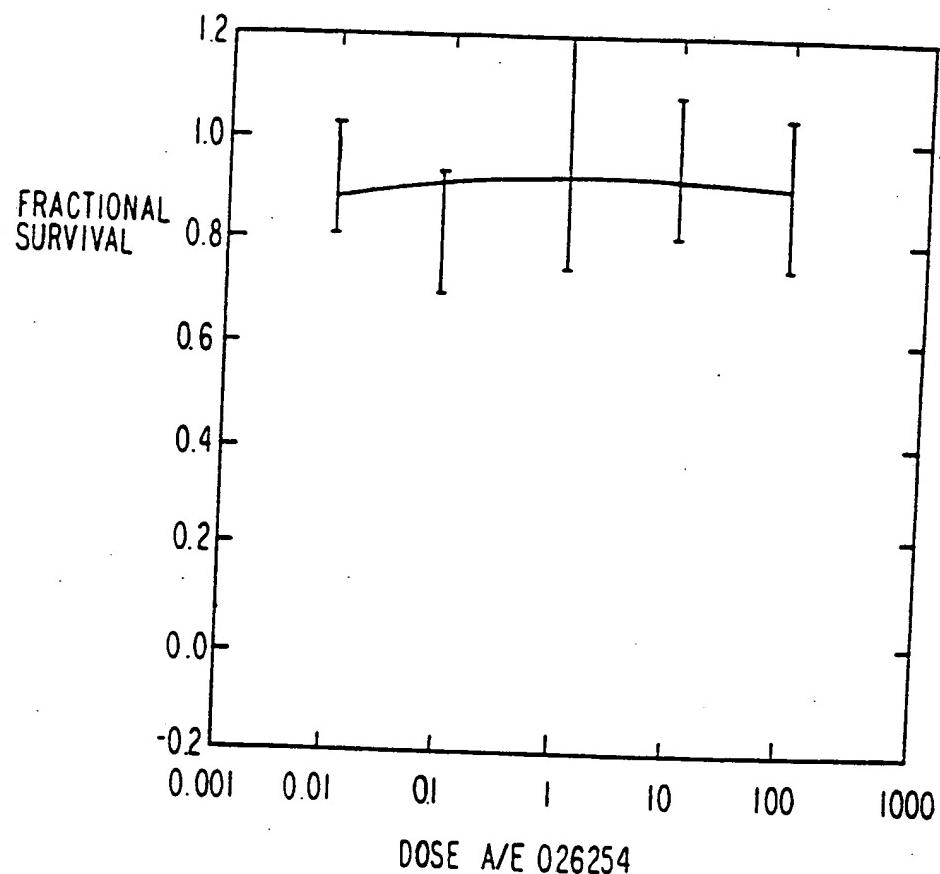
FIG. 11E



SUBSTITUTE SHEET (RULE 26)

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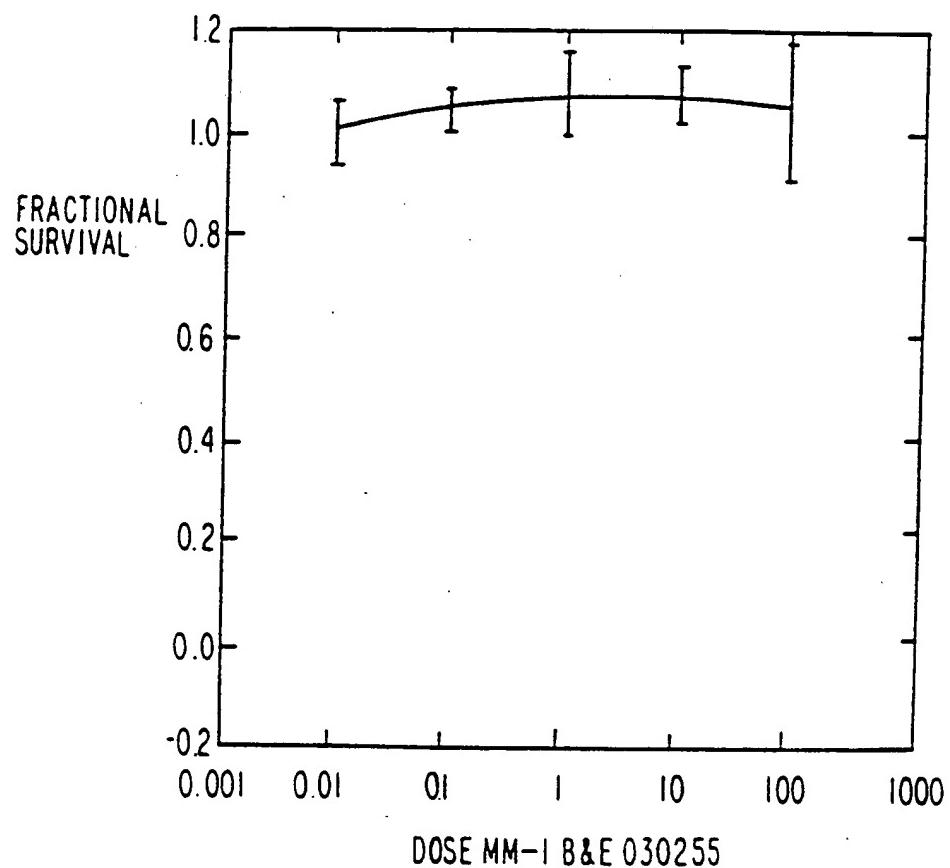
FIG. 11F



SUBSTITUTE SHEET (RULE 26)

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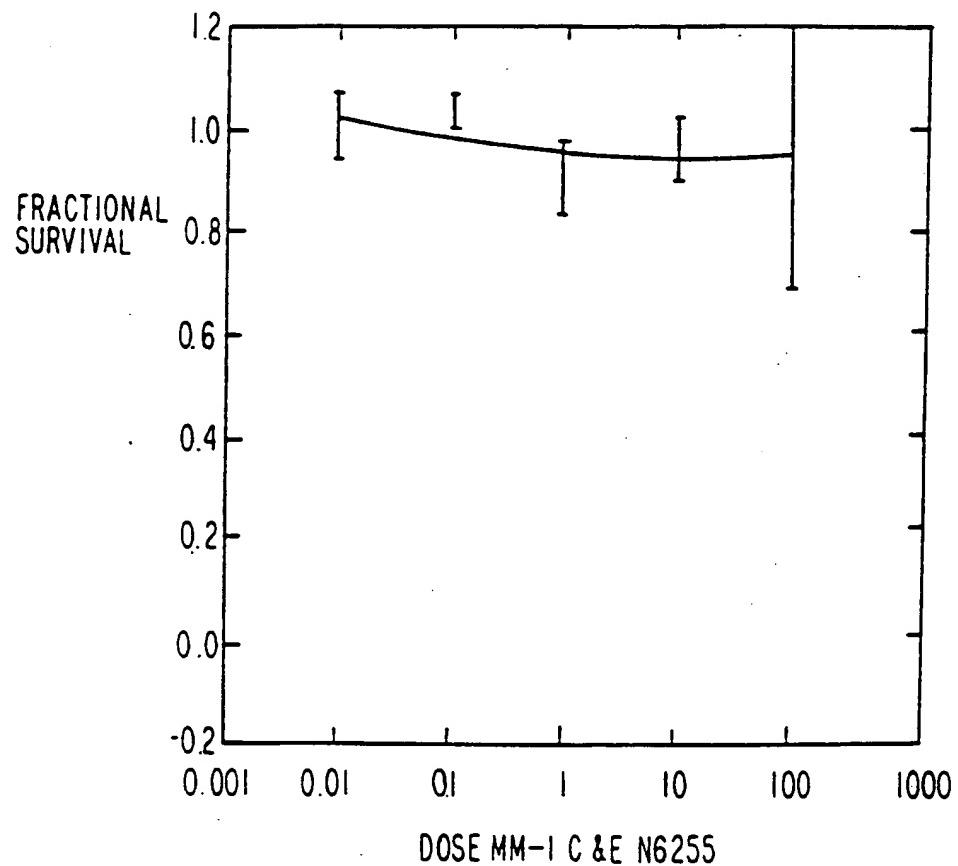
FIG. 11G



SUBSTITUTE SHEET (RULE 26)

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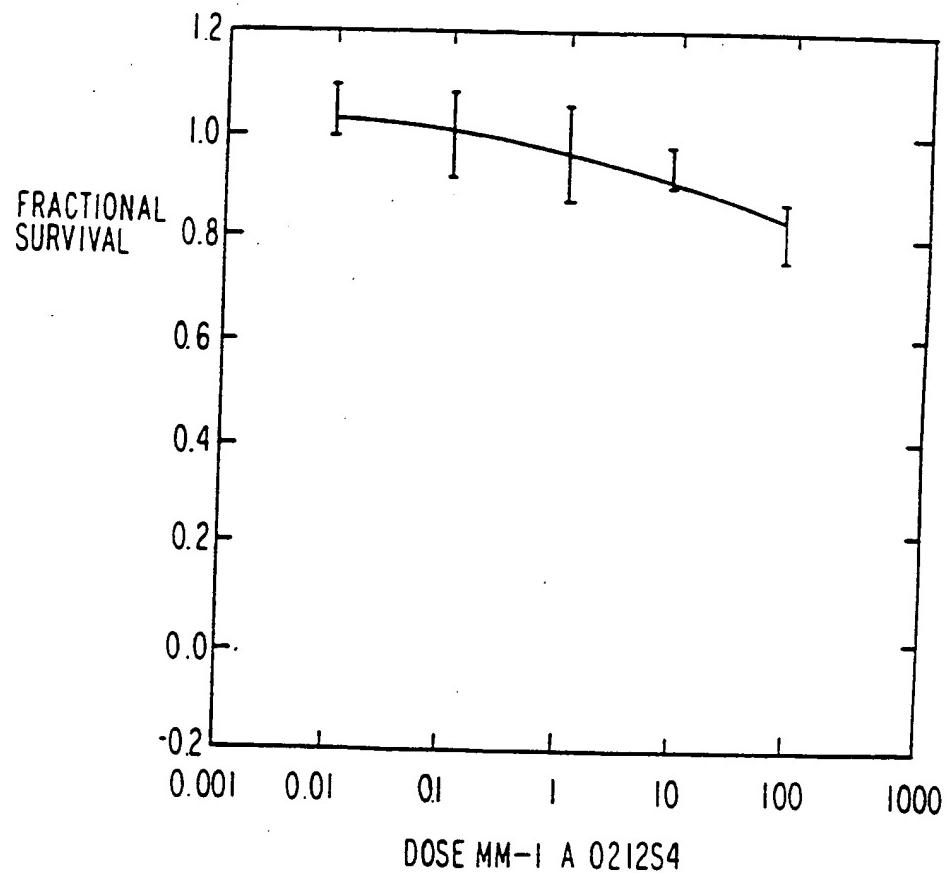
FIG. IIIH



SUBSTITUTE SHEET (RULE 26)

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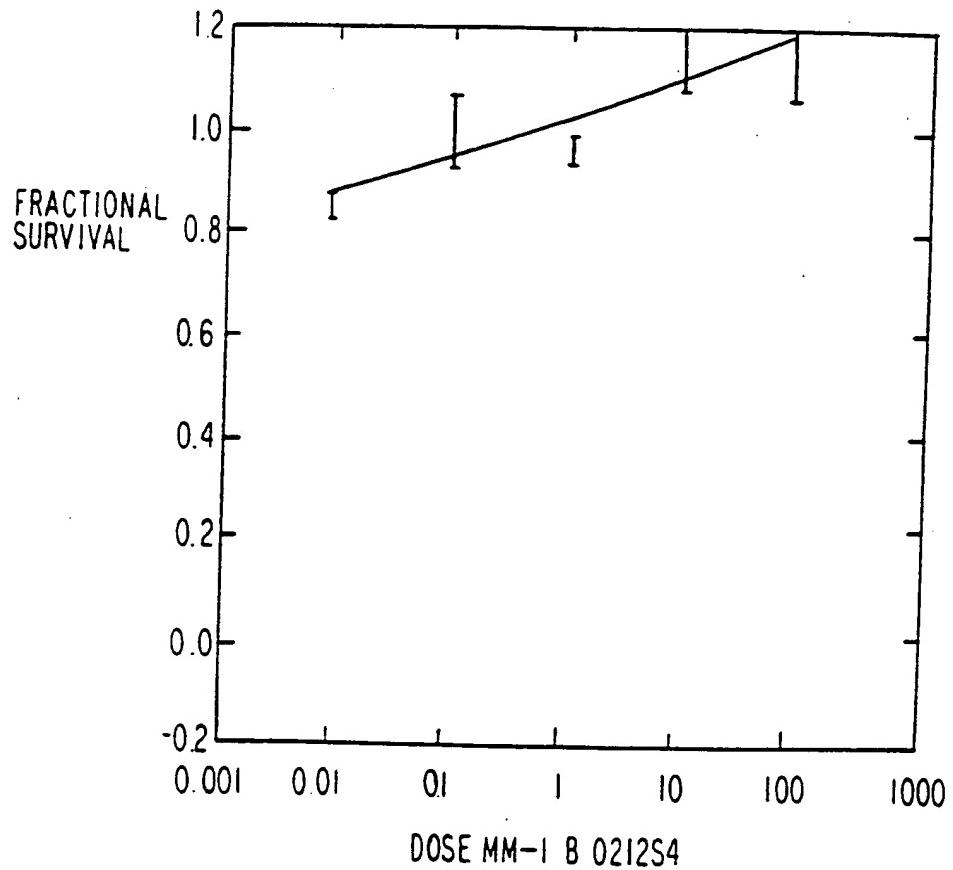
FIG. 12A



SUBSTITUTE SHEET (RULE 26)

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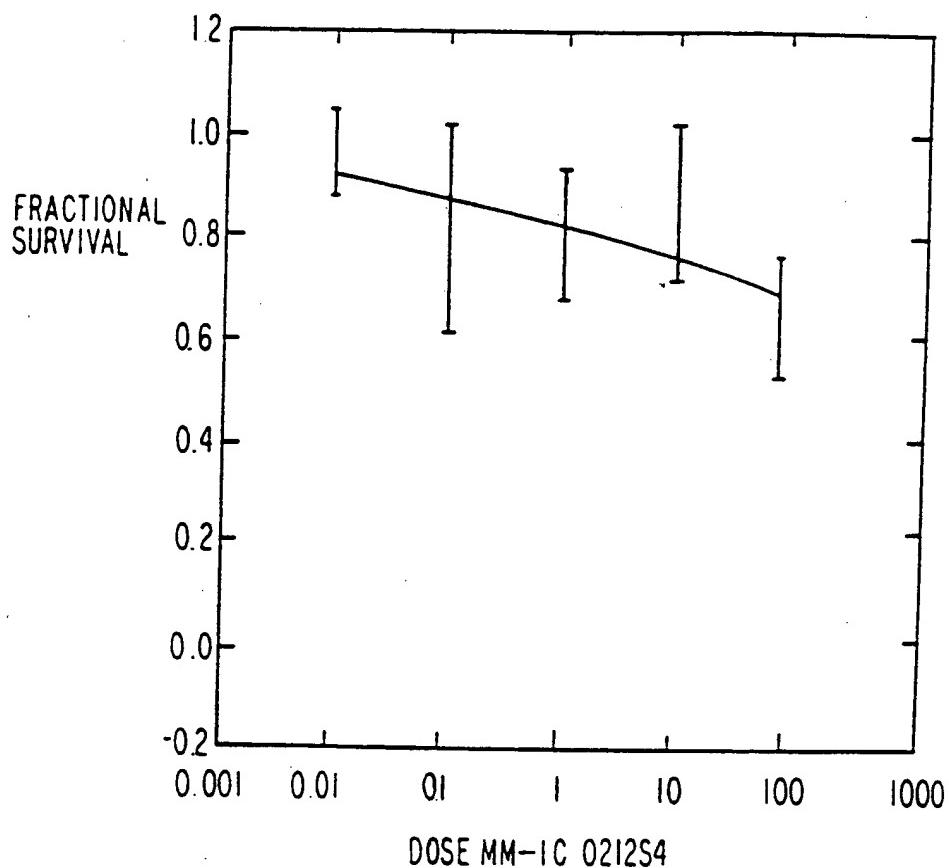
FIG. 12B



SUBSTITUTE SHEET (RULE 26)

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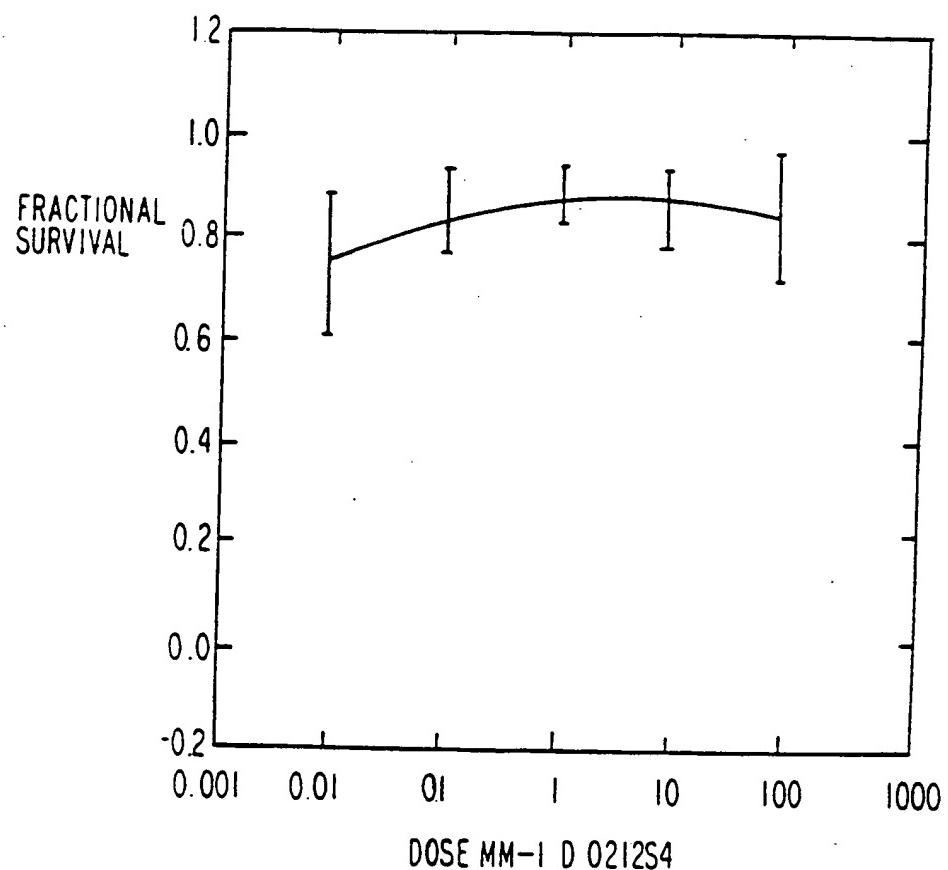
FIG. 12C



SUBSTITUTE SHEET (RULE 26)

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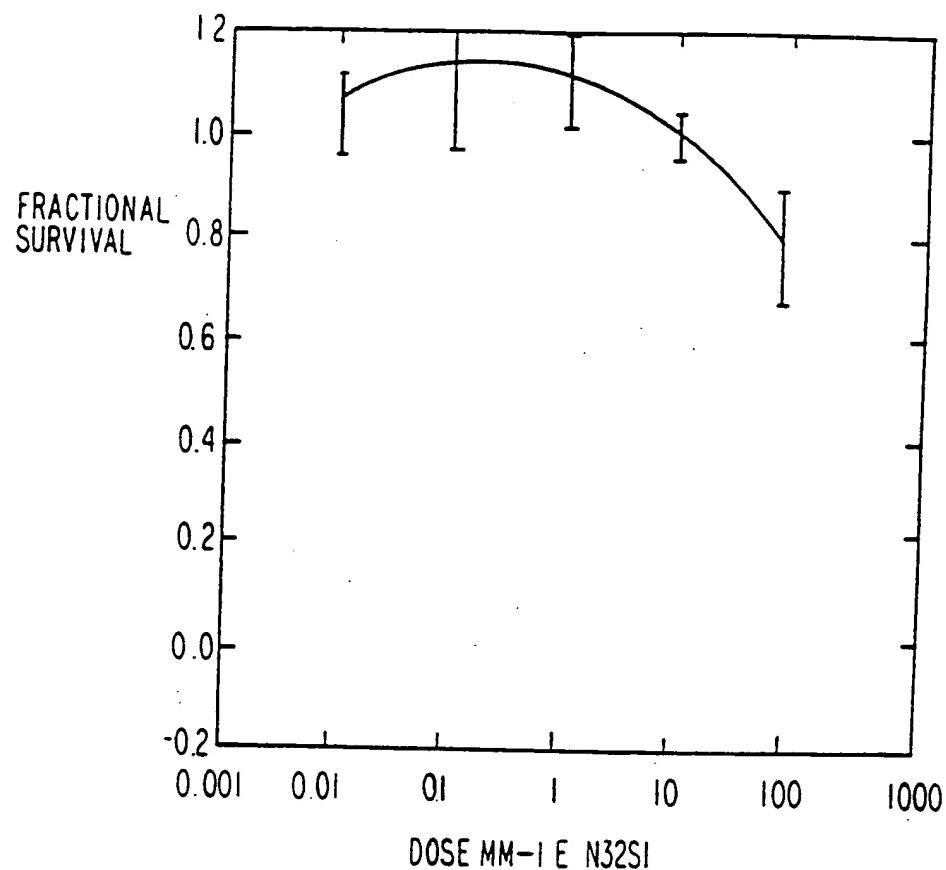
FIG. 12D



SUBSTITUTE SHEET (RULE 26)

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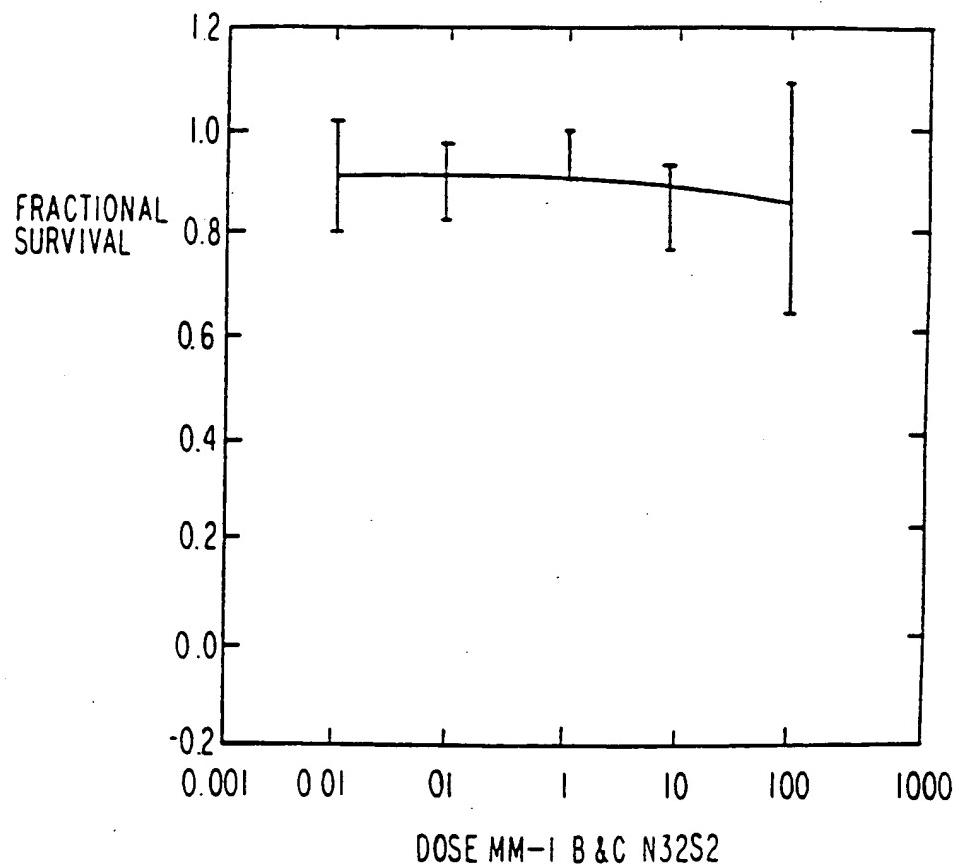
FIG. 12E



SUBSTITUTE SHEET (RULE 26)

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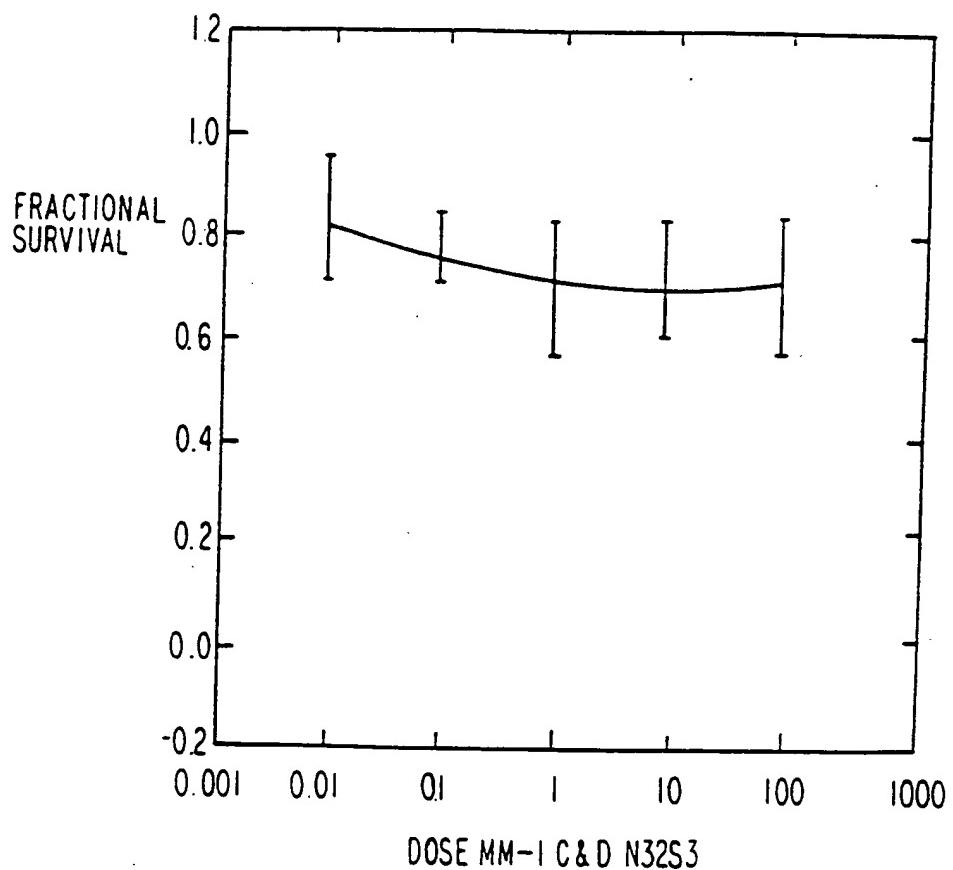
FIG. 12F



SUBSTITUTE SHEET (RULE 26)

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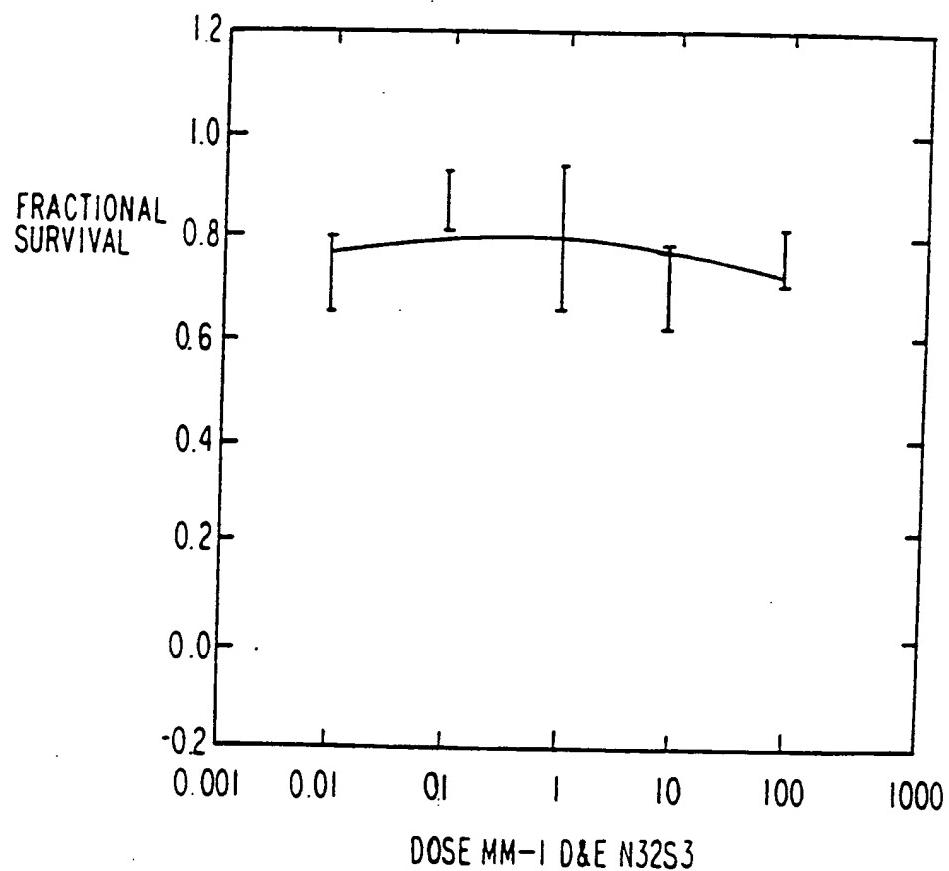
FIG. 12G



SUBSTITUTE SHEET (RULE 26)

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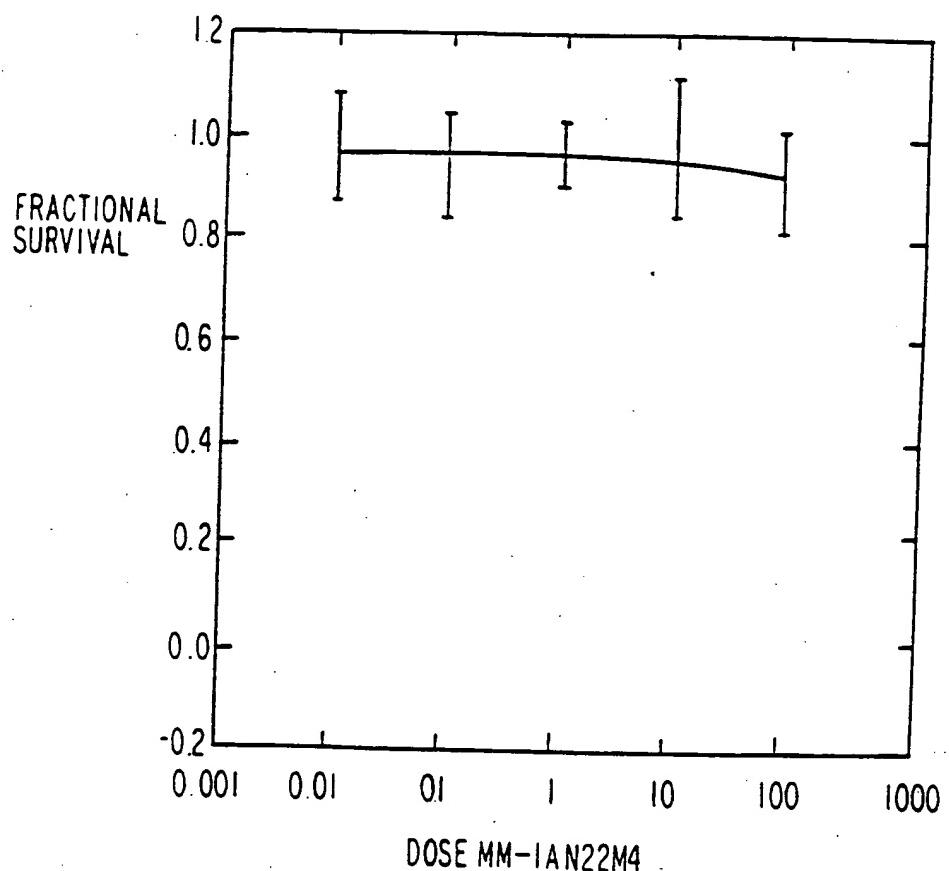
FIG. 12H



SUBSTITUTE SHEET (RULE 26)

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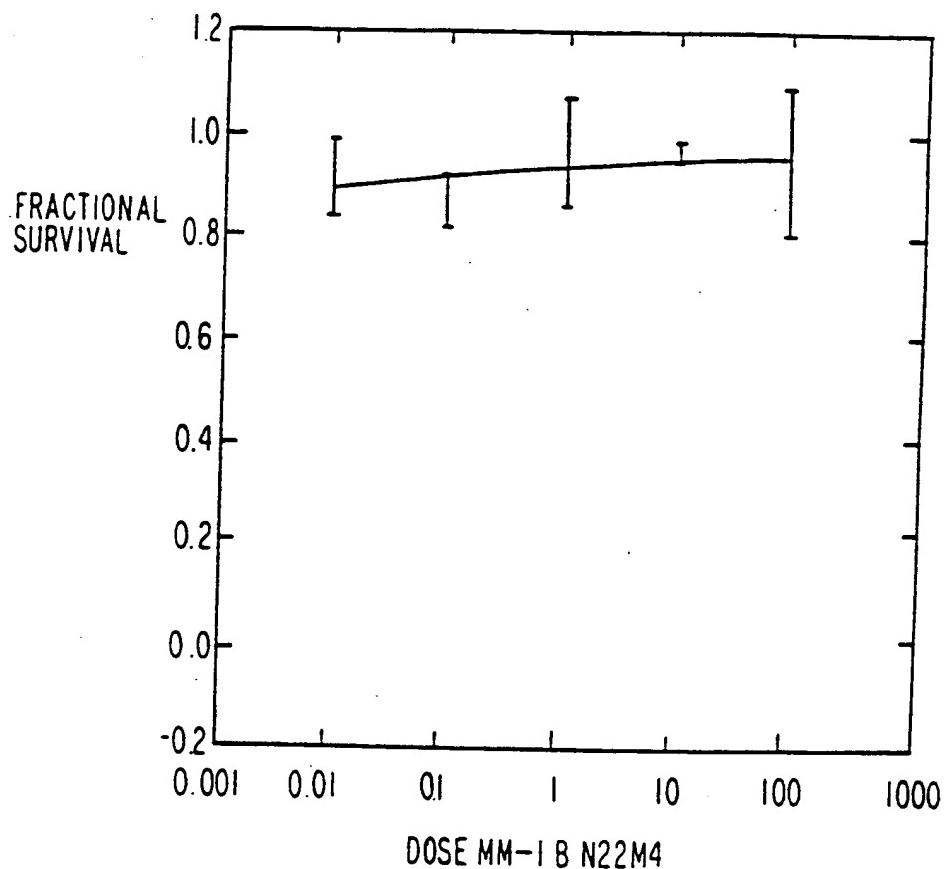
FIG. 13A



SUBSTITUTE SHEET (RULE 26)

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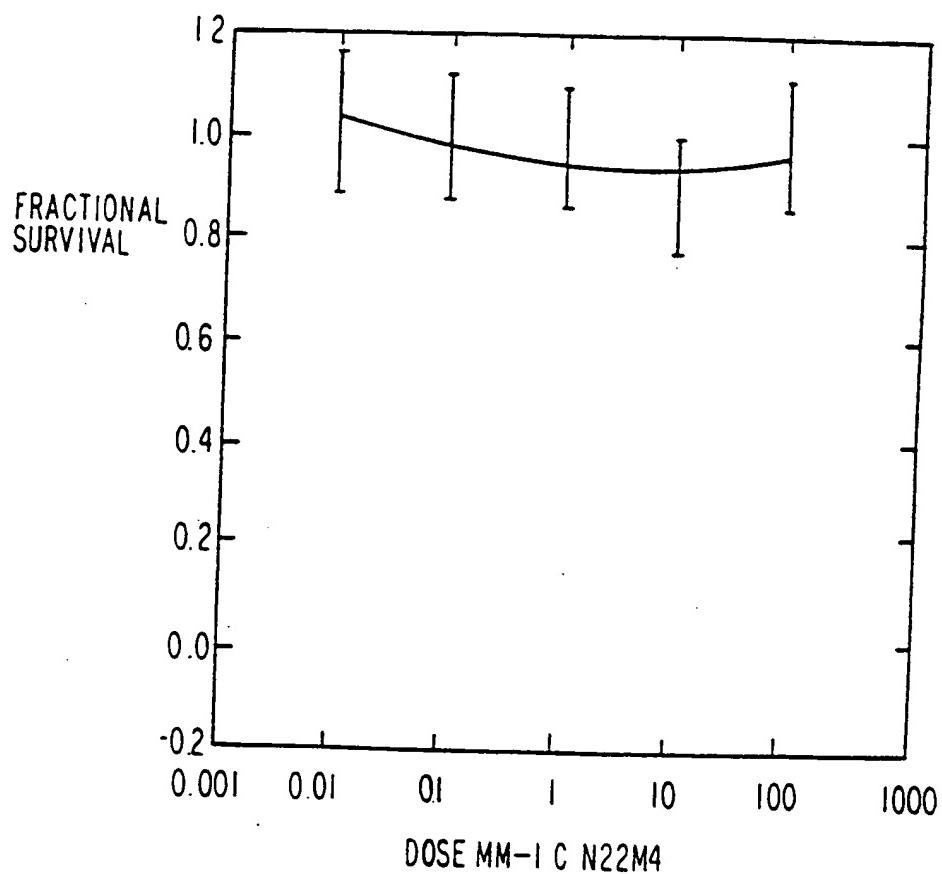
FIG. 13B



SUBSTITUTE SHEET (RULE 26)

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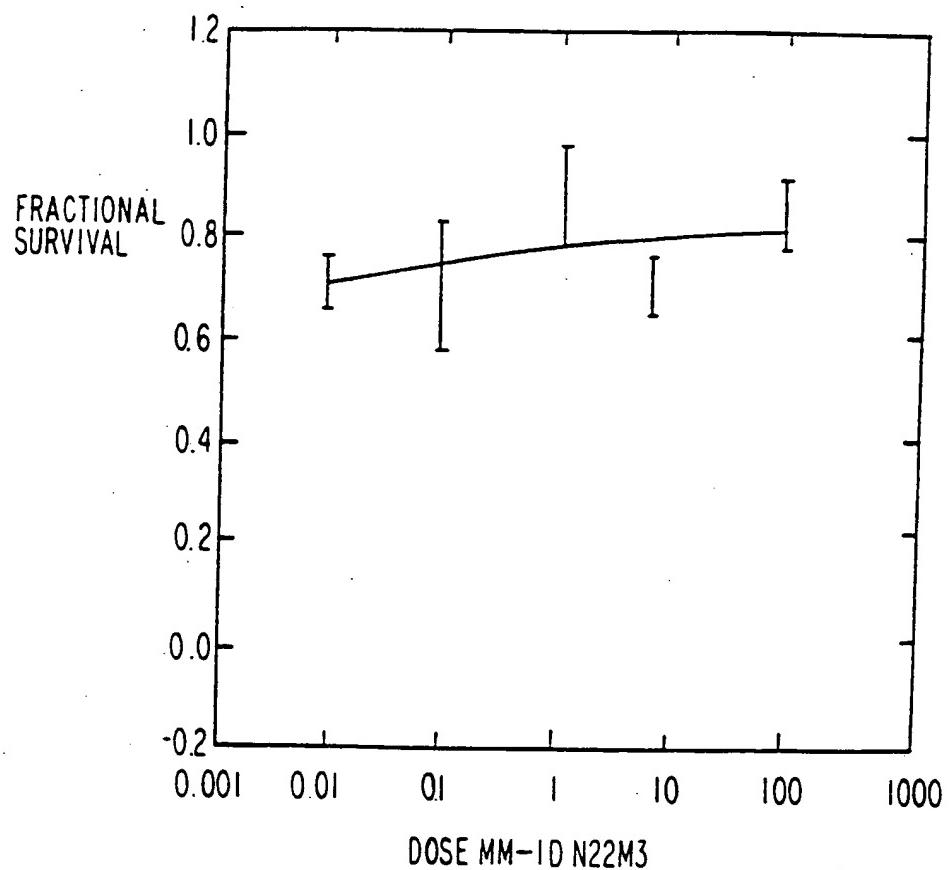
FIG. 13C



SUBSTITUTE SHEET (RULE 26)

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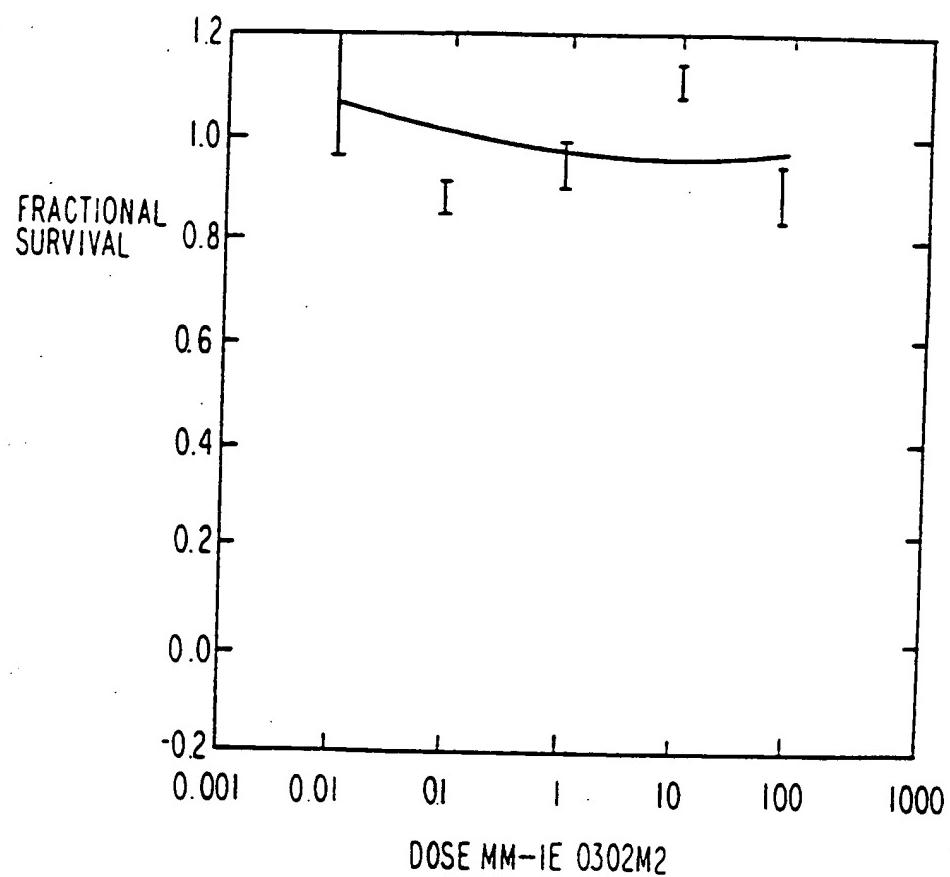
FIG. 13D



SUBSTITUTE SHEET (RULE 26)

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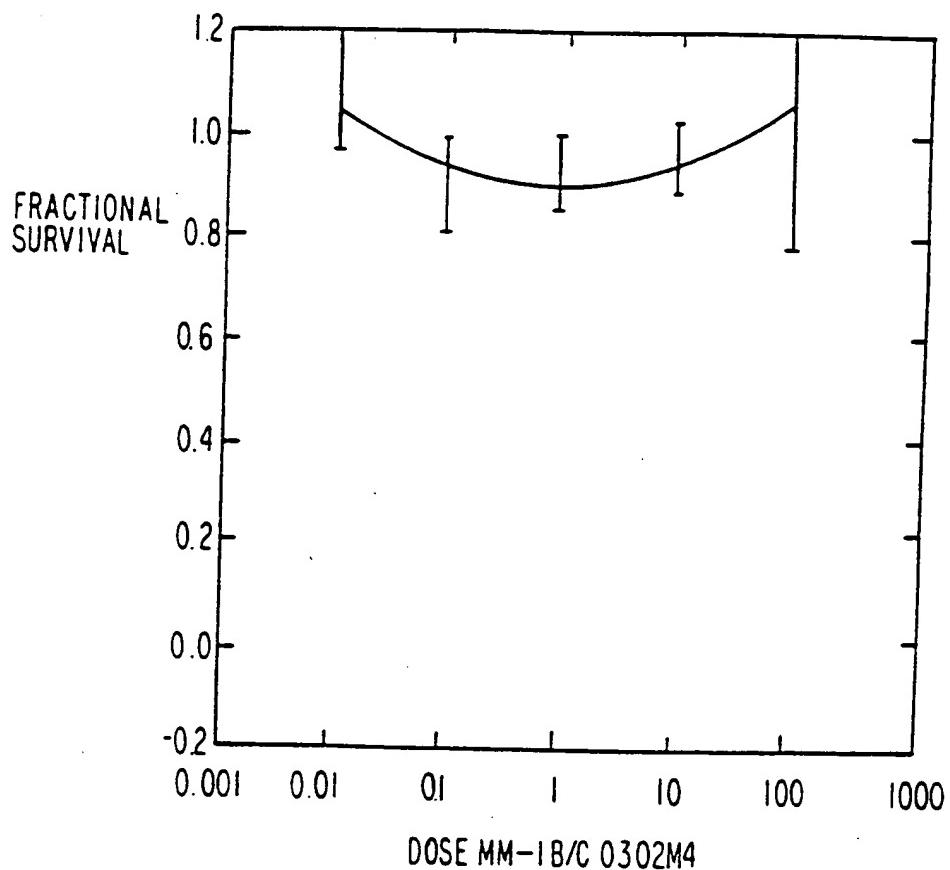
FIG. 13E



SUBSTITUTE SHEET (RULE 26)

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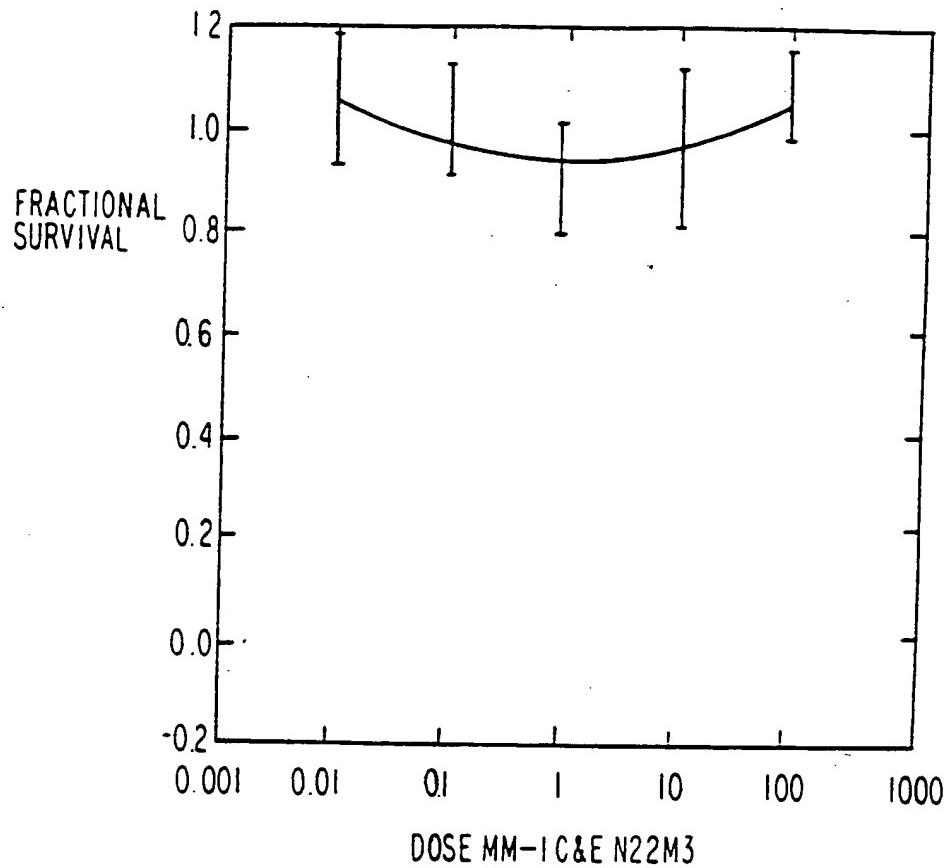
FIG. 13F



SUBSTITUTE SHEET (RULE 26)

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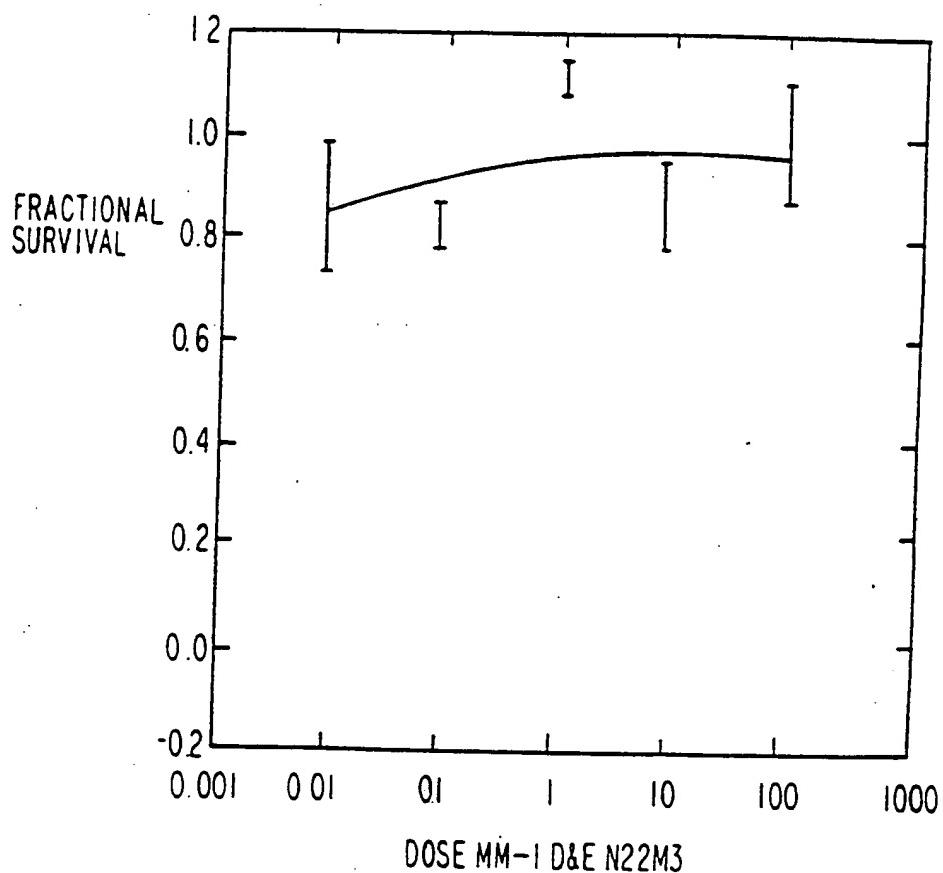
FIG. 13G



SUBSTITUTE SHEET (RULE 26)

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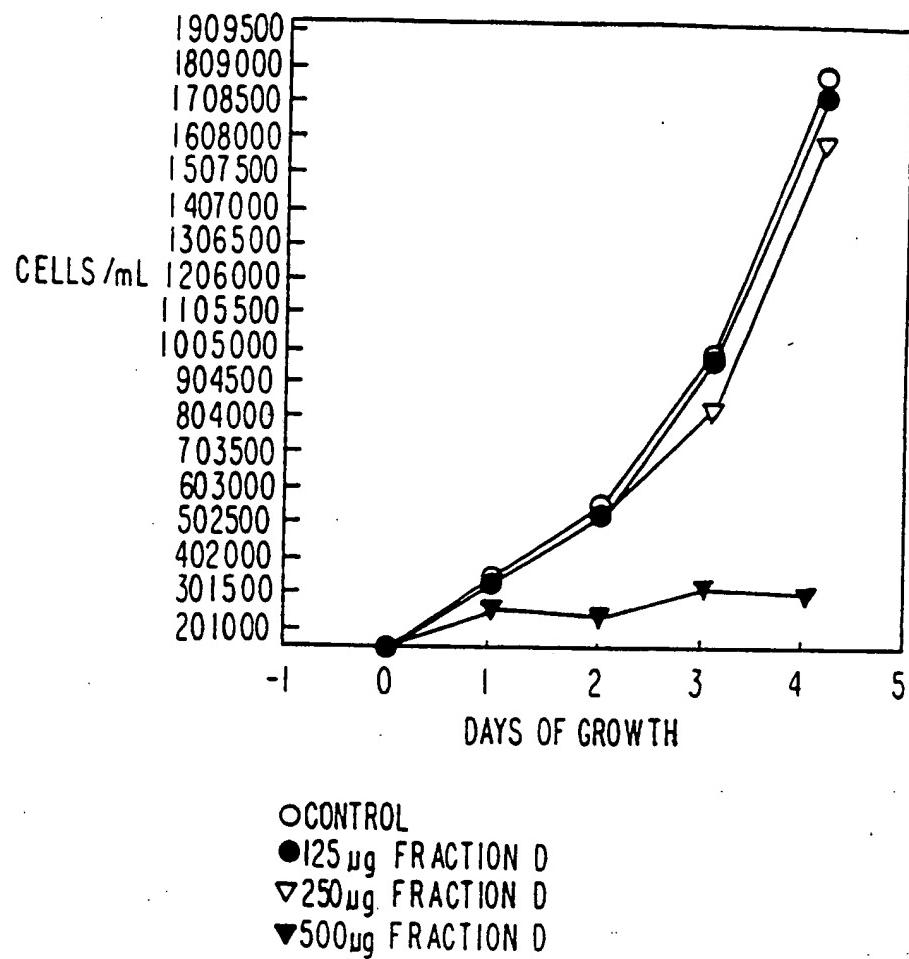
FIG. 13H



SUBSTITUTE SHEET (RULE 26)

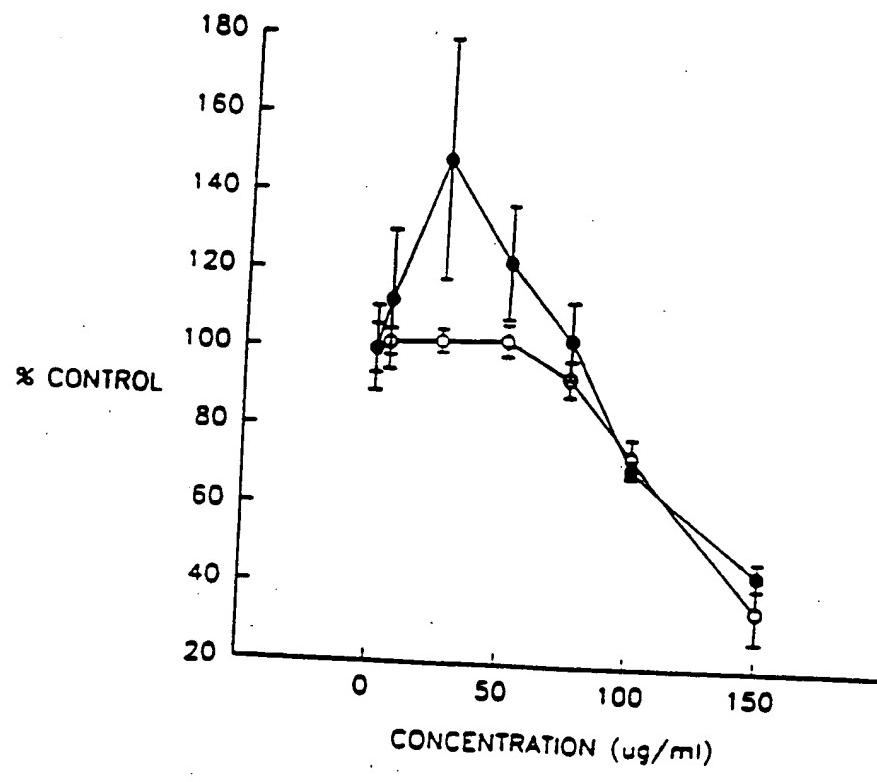
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FIG. 14



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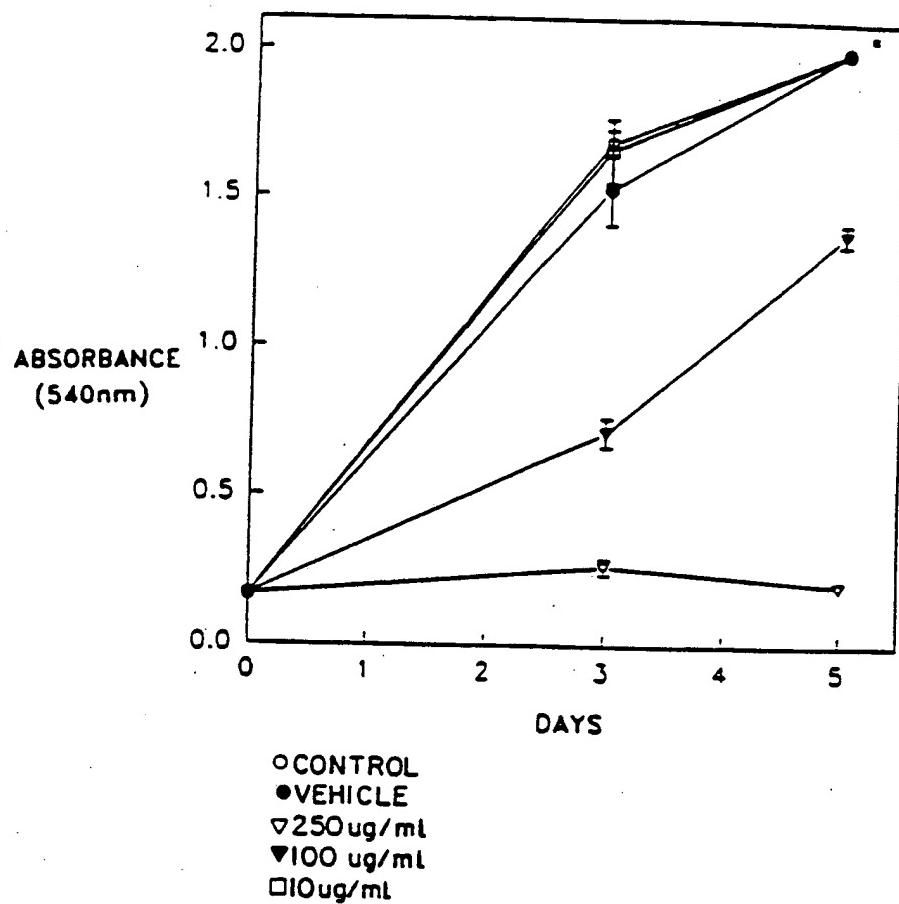
FIG. 15A



- XTT STAINING
- CRYSTAL VIOLET STAINING

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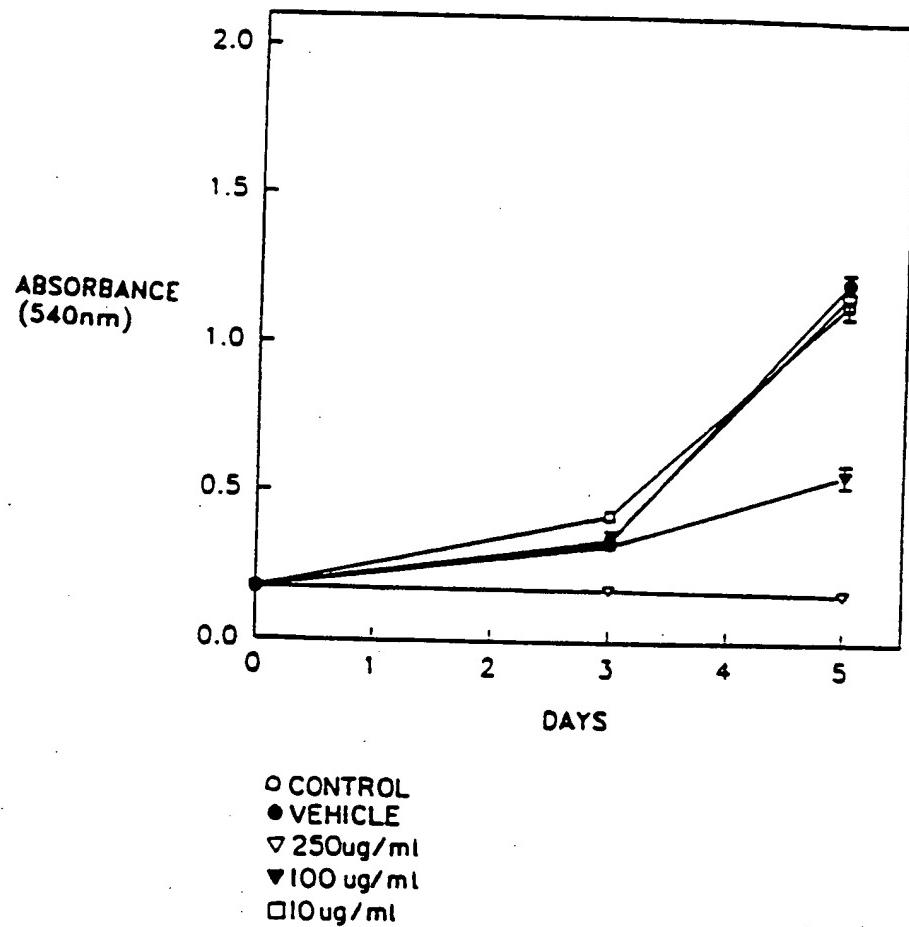
FIG. 15B



* NOTE: ABSORBANCE OF 2.0 INDICATES THE MAXIMUM ABSORBANCE OF THE PLATE READER. IT IS NOT REPRESENTATIVE OF CELL NUMBER.

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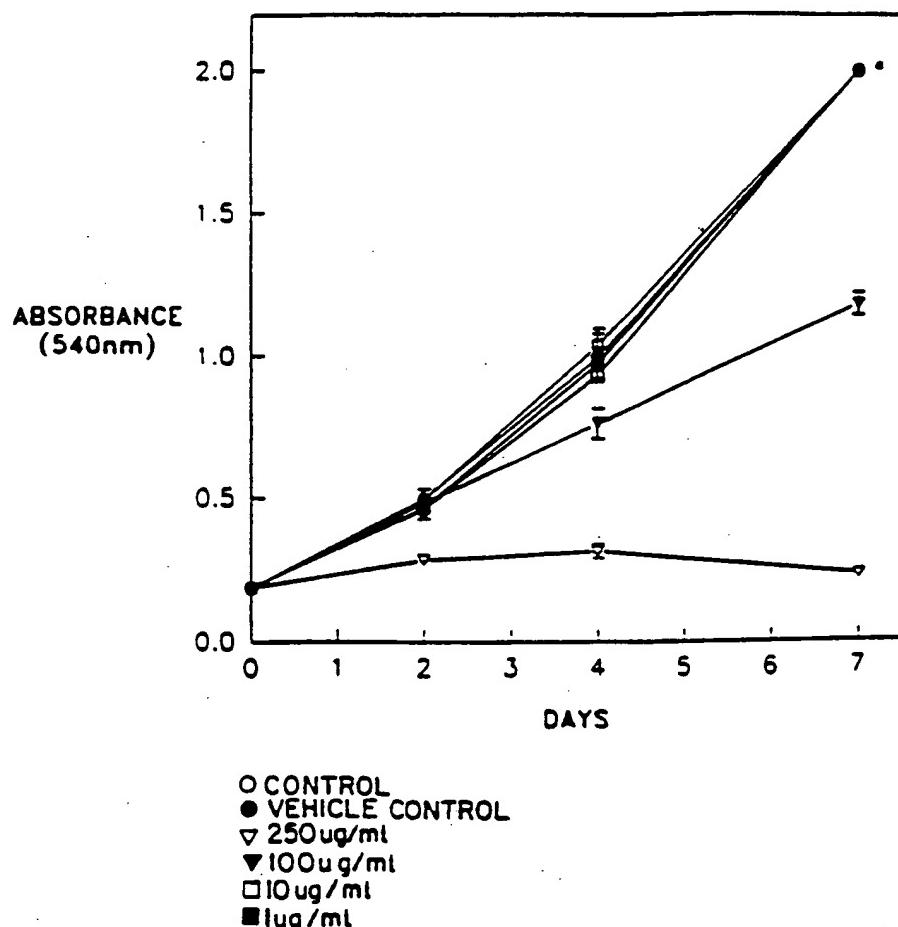
FIG. 15C



SUBSTITUTE SHEET (RULE 26)

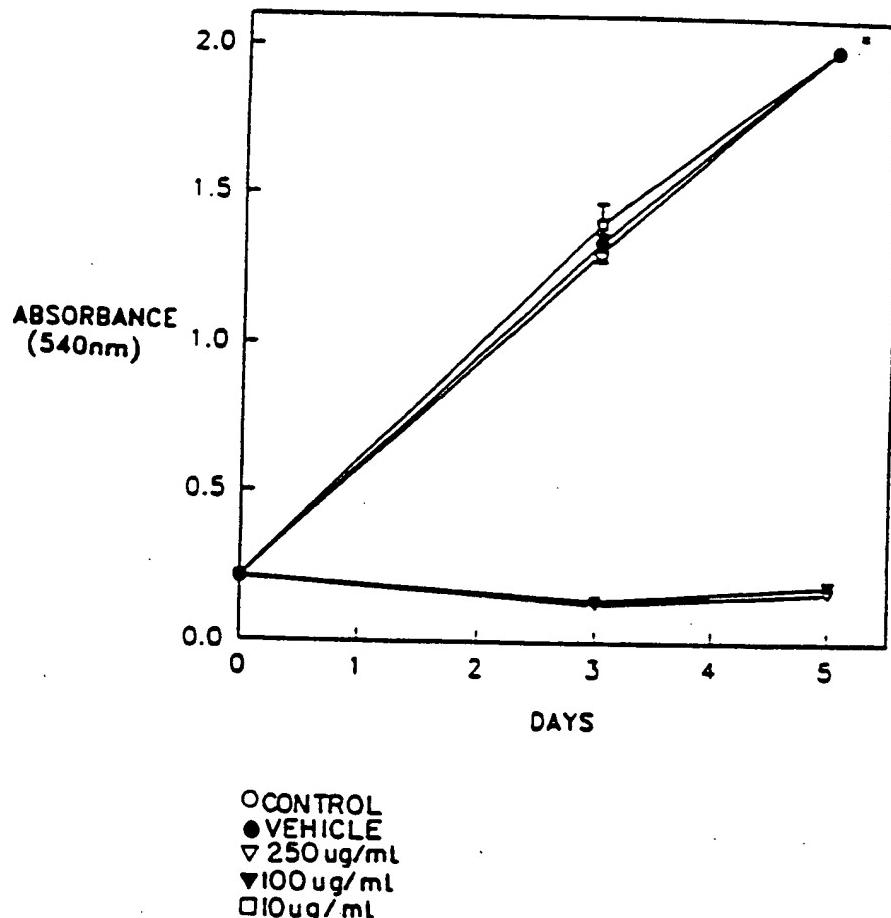
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FIG. 15D



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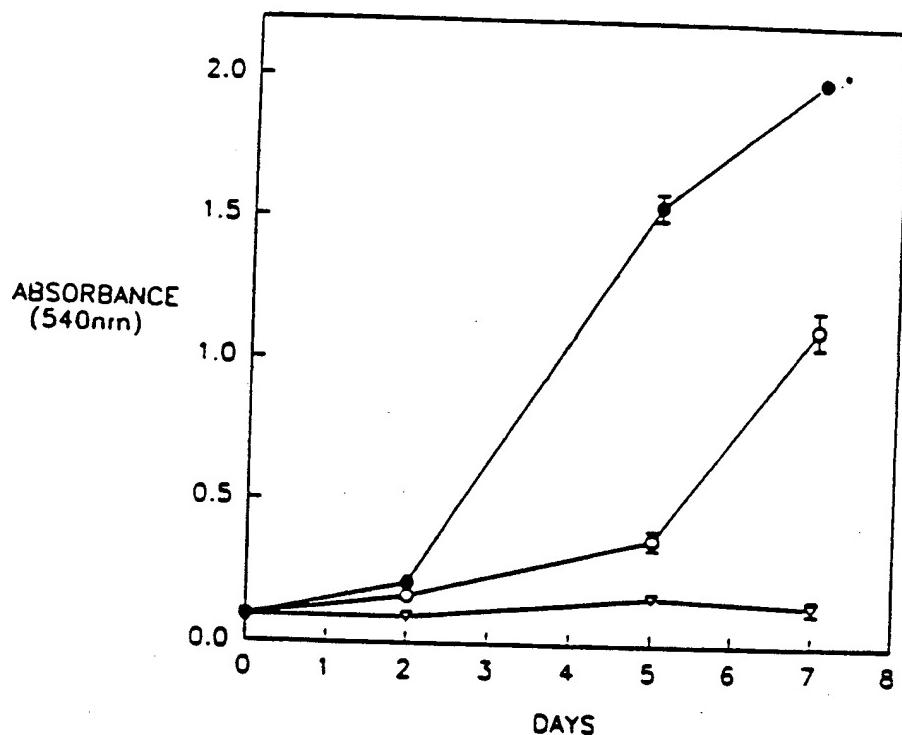
FIG. 15E



* NOTE: ABSORBANCE OF 2.0 INDICATES THE MAXIMUM ABSORBANCE OF THE PLATE READER. IT IS NOT REPRESENTATIVE OF CELL NUMBER.

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FIG. 15F



○ 100µg/ml FRACTIONS A-E (#64)

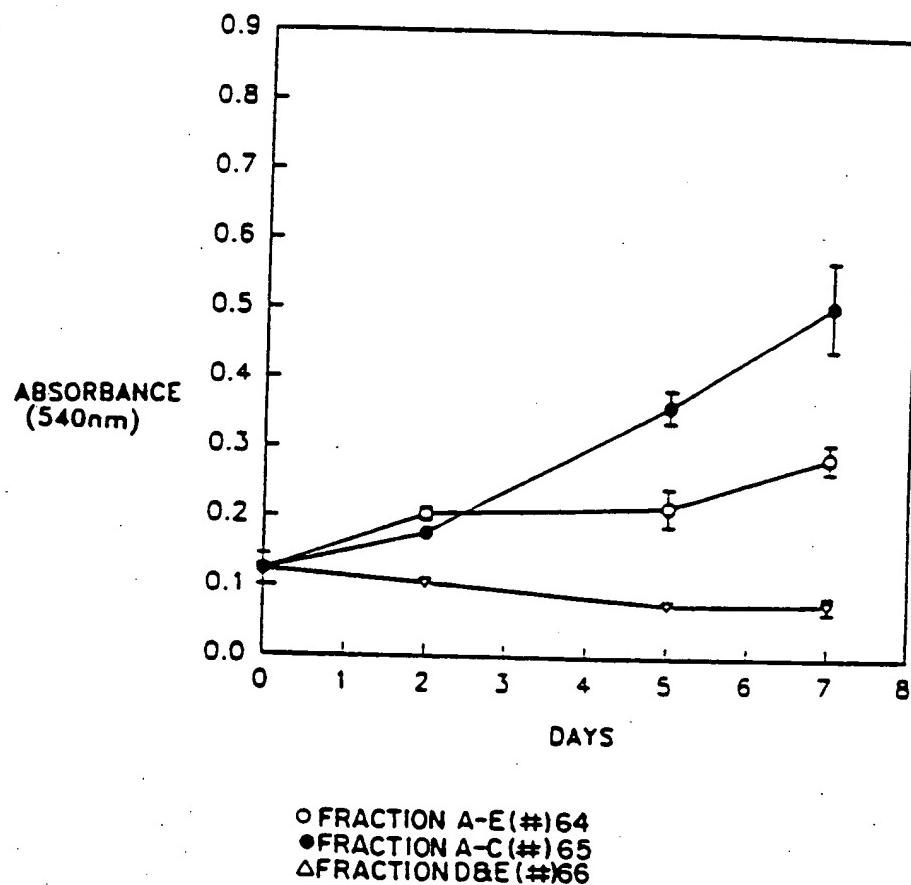
● 100µg/ml FRACTIONS A-C (#65)

△ 100µg/ml FRACTIONS D&E (#66)

* NOTE: ABSORBANCE OF 2.0 INDICATES THE MAXIMUM
ABSORBANCE OF THE PLATE READER. IT IS NOT
REPRESENTATIVE OF CELL NUMBER.

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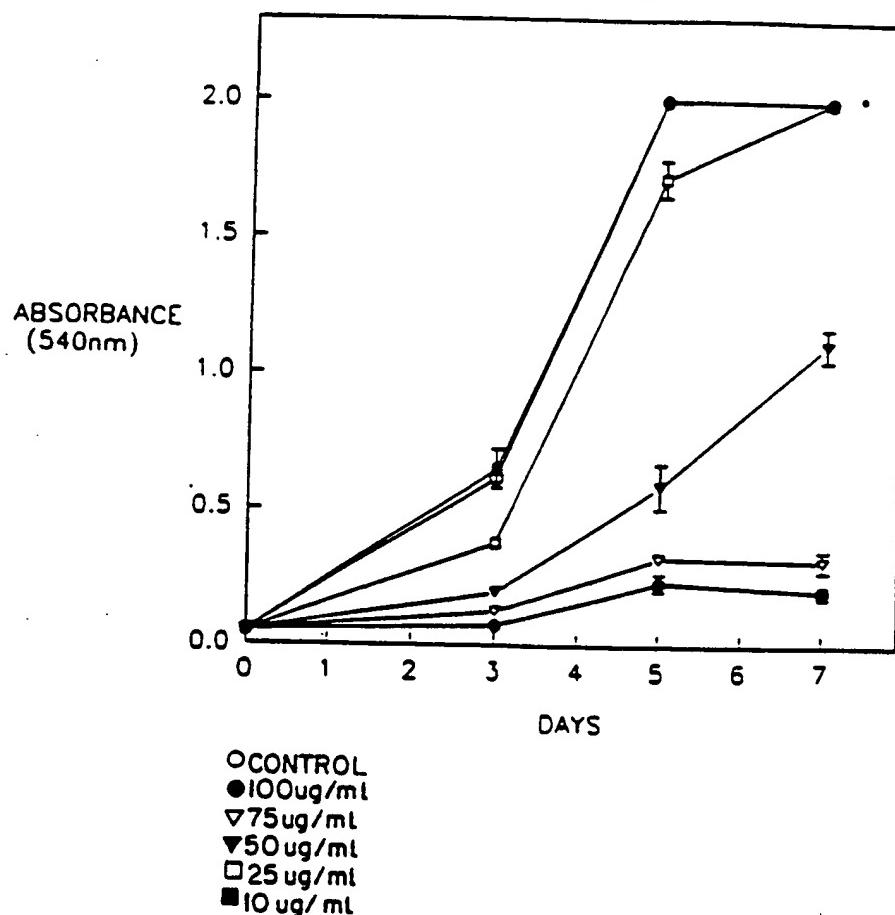
FIG. 15G



SUBSTITUTE SHEET (RULE 26)

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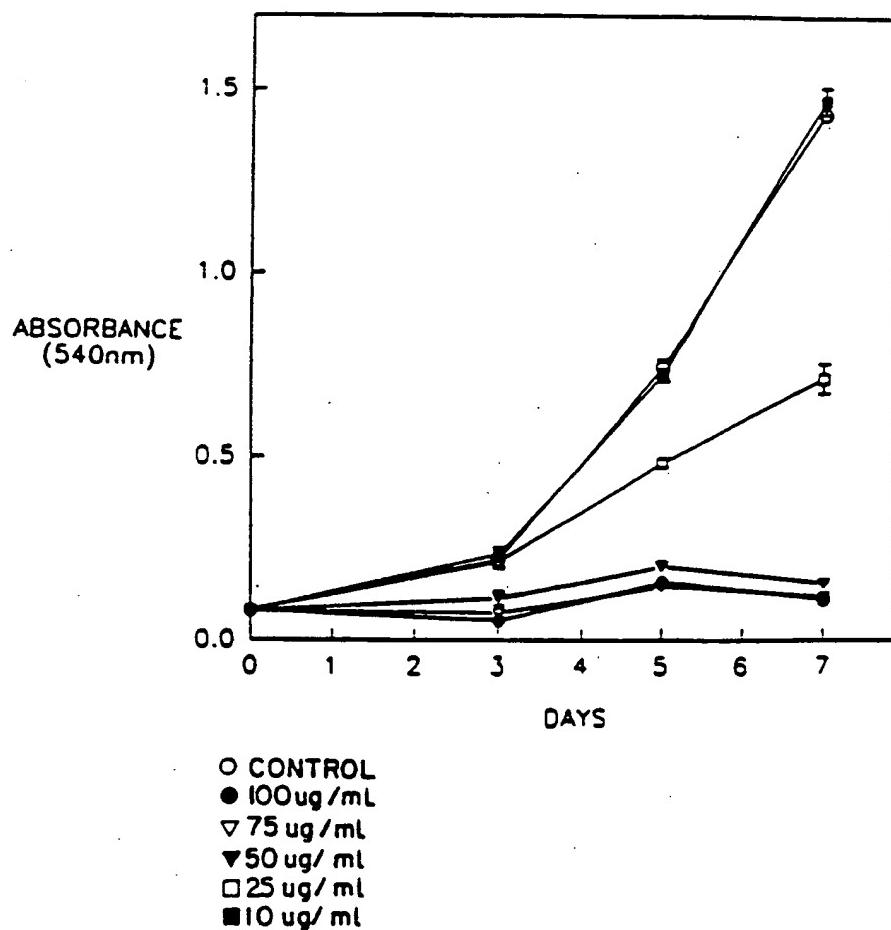
FIG. 15H



*NOTE: ABSORBANCE OF 2.0 INDICATES THE MAXIMUM ABSORBANCE OF THE PLATE READER. IT IS NOT REPRESENTATIVE OF CELL NUMBER.

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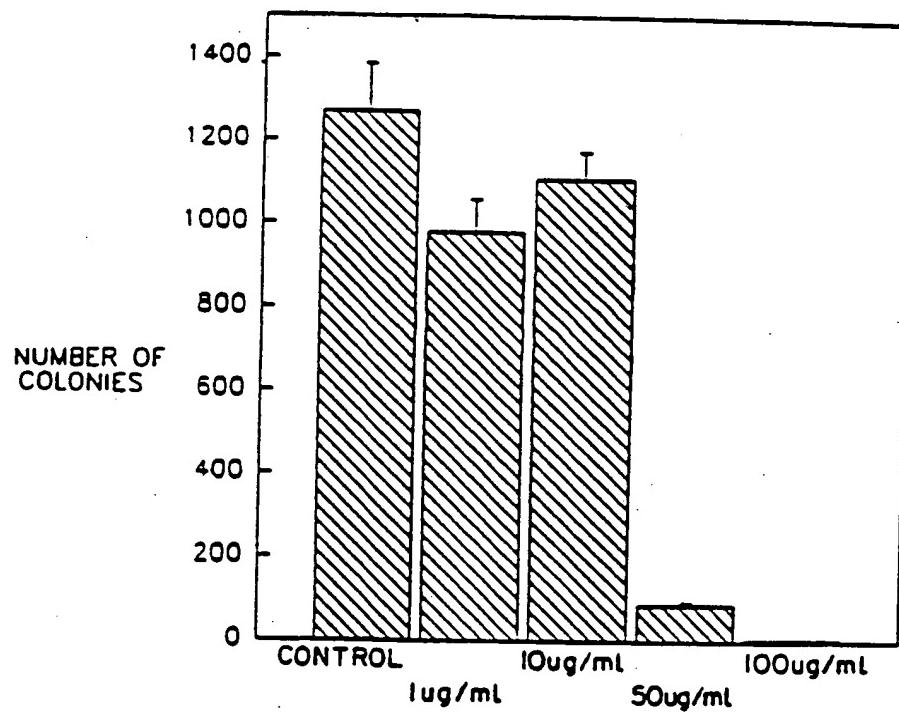
FIG. 15I



SUBSTITUTE SHEET (RULE 26)

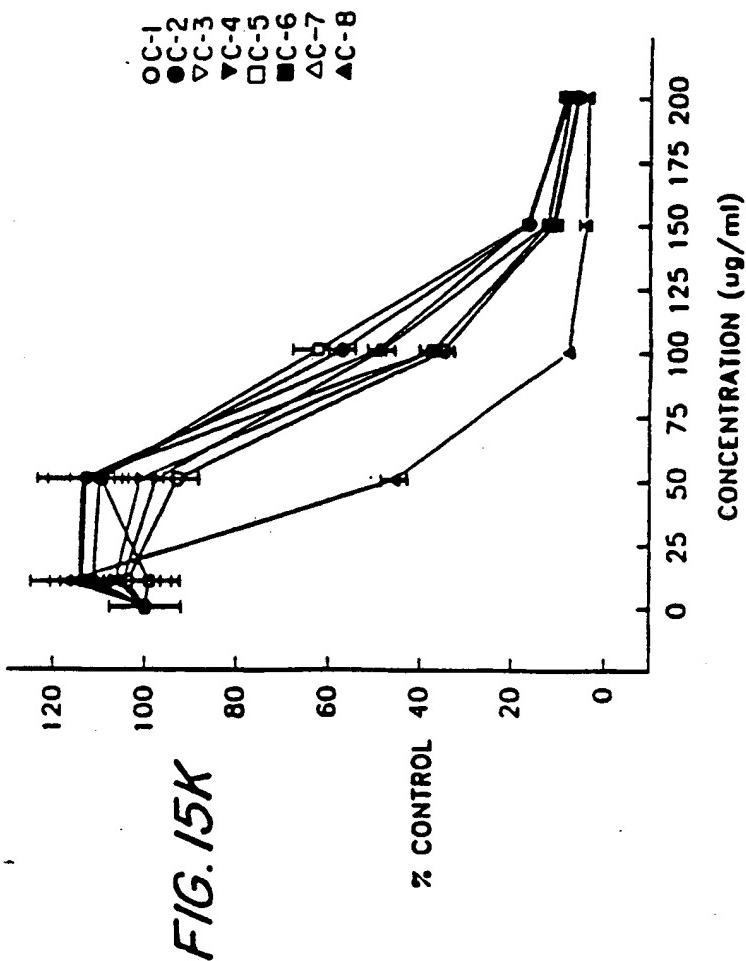
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FIG. 15J



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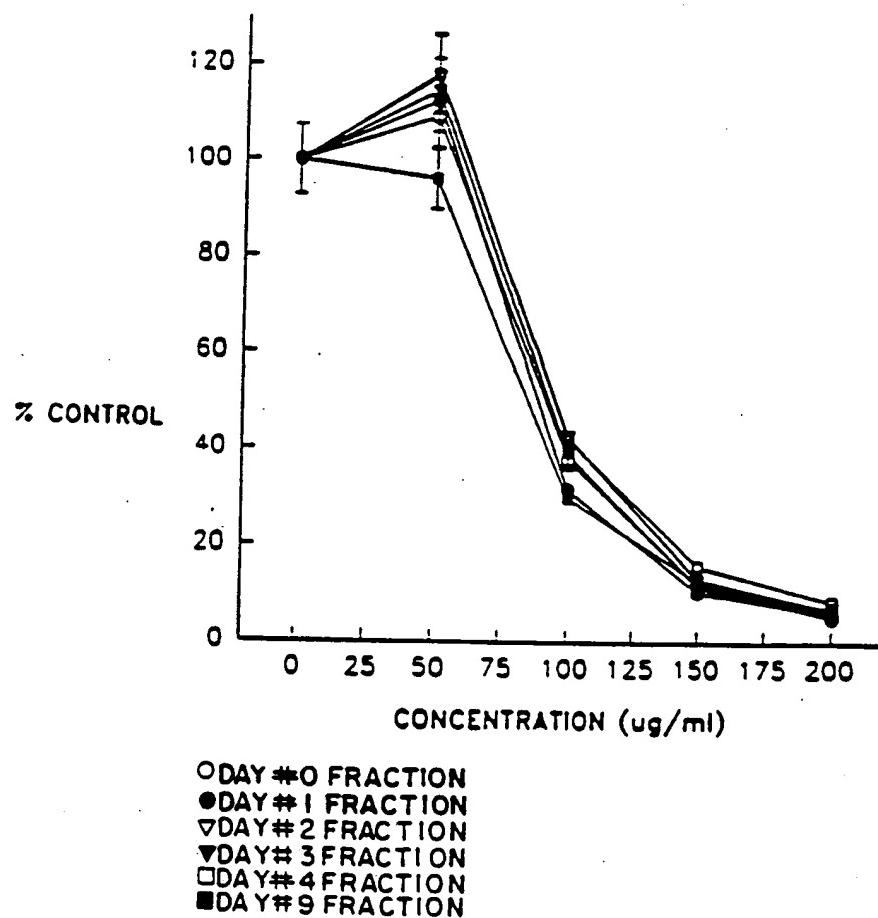


# GENOTYPE	HORTI, RACE	DESCRIPTION
C-1 UF-12	CRIOULLO	CRUDE EXTRACTS OF UF-12(BRAZIL) COCOA POLYPHENOLS (DECAFFEINATED/DE THREOBROMINATED)
C-2 NA-33	FORASTERO	CRUDE EXTRACTS OF NA-33 (BRAZIL) COCOA POLYPHENOLS (DECAFFEINATED/DE THREOBROMINATED)
C-3 FEC-48	FORASTERO	CRUDE EXTRACTS OF FEC-48 (BRAZIL) COCOA POLYPHENOLS (DECAFFEINATED/DE THREOBROMINATED)
C-4 UNKNOWN	FORASTERO	CRUDE EXTRACTS OF UNKNOWN (W. AFRICA) COCOA POLYPHENOLS (DECAFFEINATED/DE THREOBROMINATED)
C-5 UF-613(BRAZIL)	TRINITARIO	CRUDE EXTRACTS OF UF-613(BRAZIL) COCOA POLYPHENOLS (DECAFFEINATED/DE THREOBROMINATED)
C-6 ICS-100	TRINITARIO	CRUDE EXTRACTS OF ICS-100(BRAZIL) COCOA POLYPHENOLS (DECAFFEINATED/DE THREOBROMINATED)
C-7 ICS-139	TRINITARIO	CRUDE EXTRACTS OF ICS-139 (BRAZIL) COCOA POLYPHENOLS (DECAFFEINATED/DE THREOBROMINATED)
C-8 UTT-1	TRINITARIO	CRUDE EXTRACTS OF UTT-1 (MALAYSIA) COCOA POLYPHENOLS (DECAFFEINATED/DE THREOBROMINATED)

SUBSTITUTE SHEET (RULE 26)

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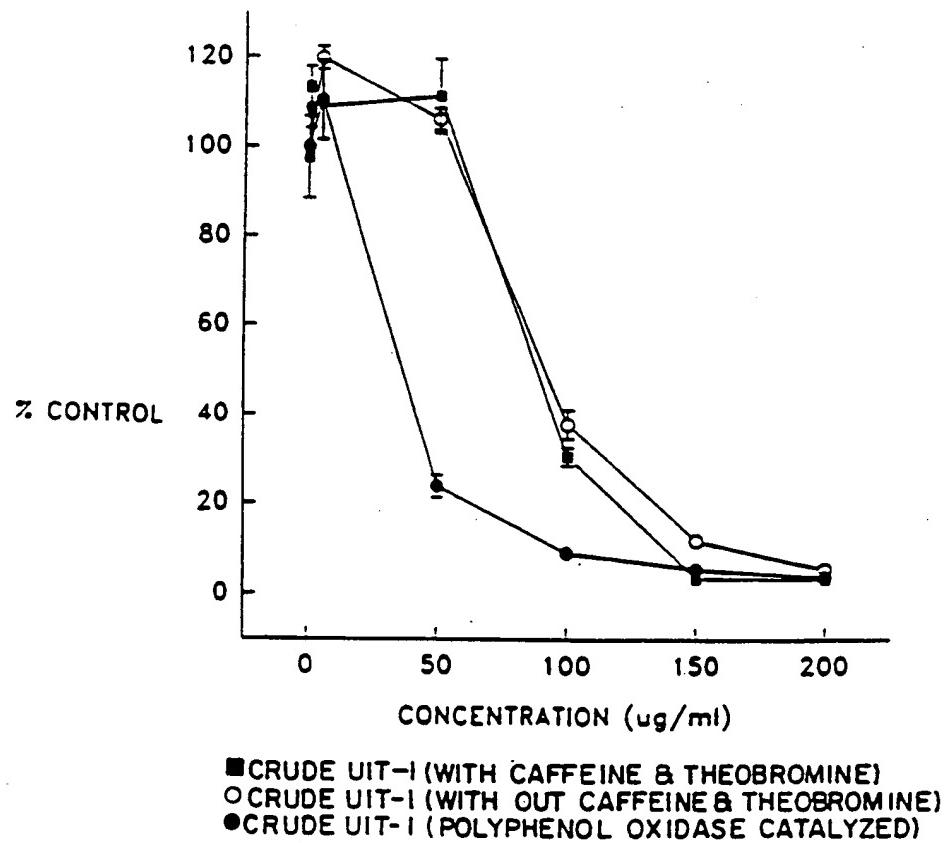
FIG. 15L



SUBSTITUTE SHEET (RULE 26)

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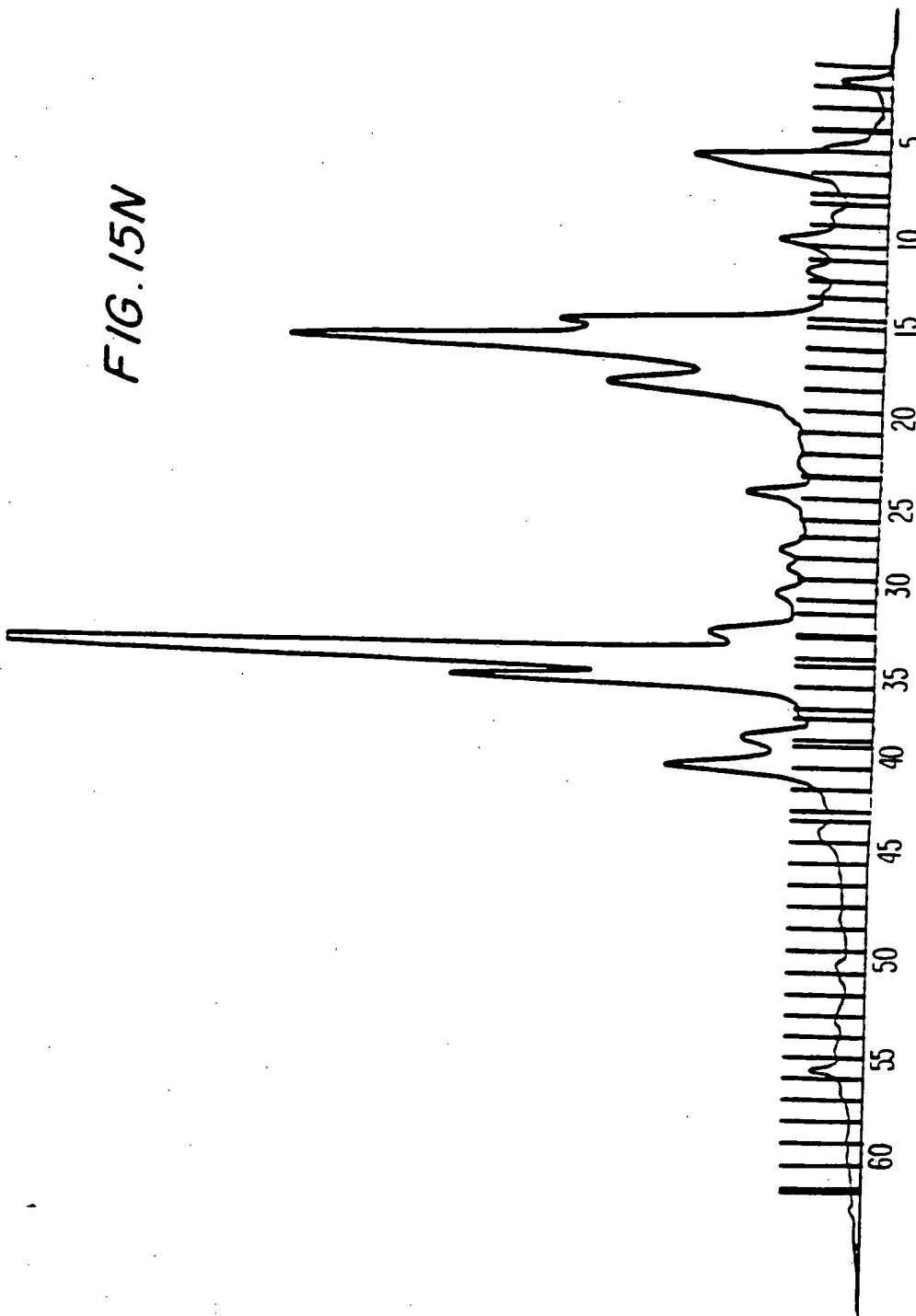
FIG. 15M



SUBSTITUTE SHEET (RULE 26)

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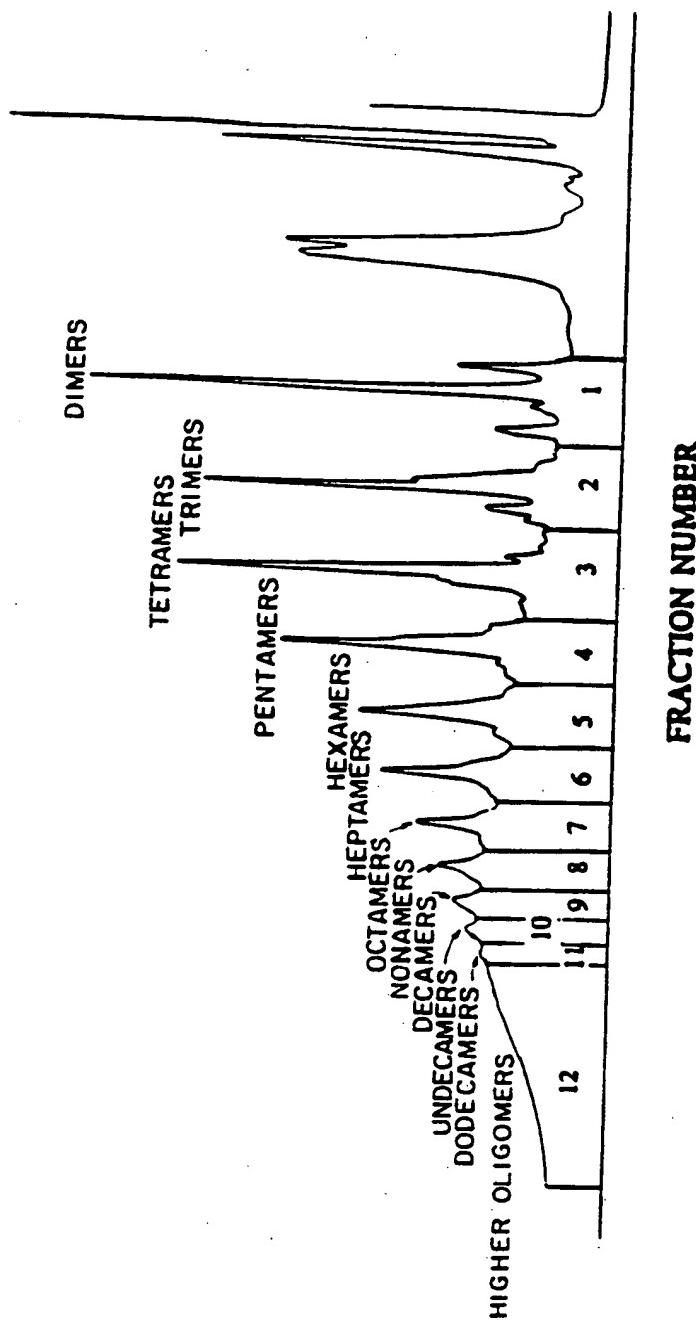
FIG. 15N



SUBSTITUTE SHEET (RULE 26)

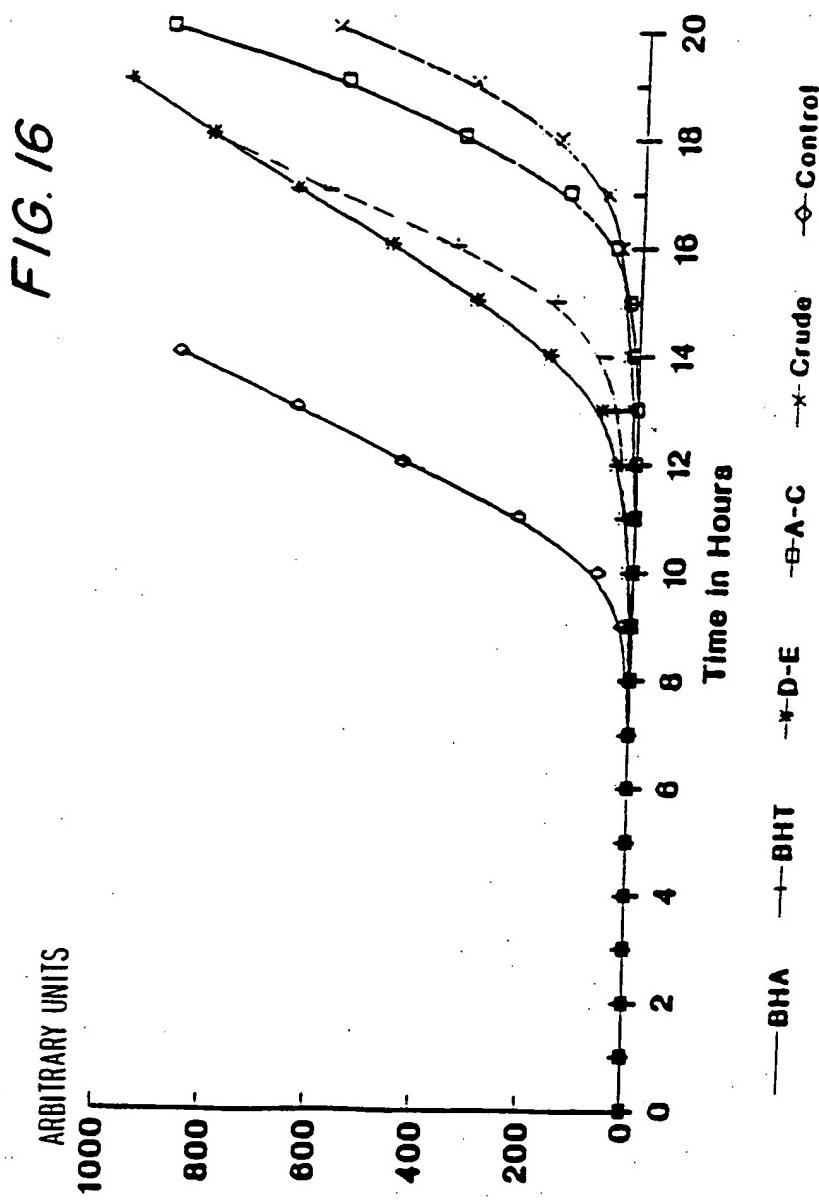
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FIG. 150



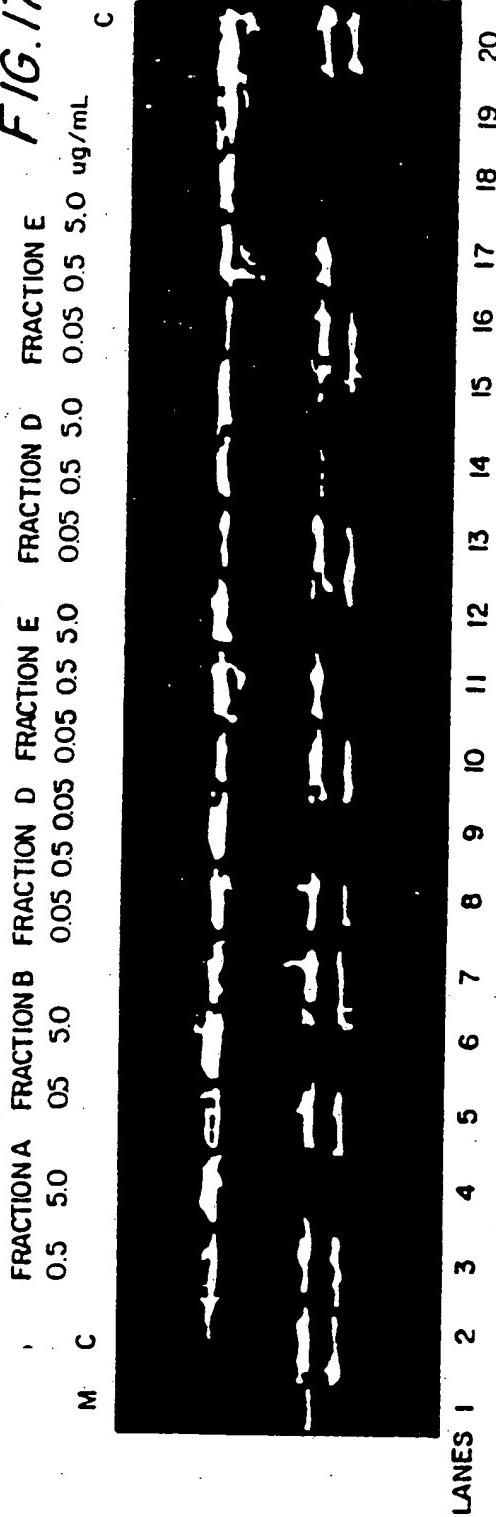
SUBSTITUTE SHEET (RULE 26)

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SUBSTITUTE SHEET (RULE 26)

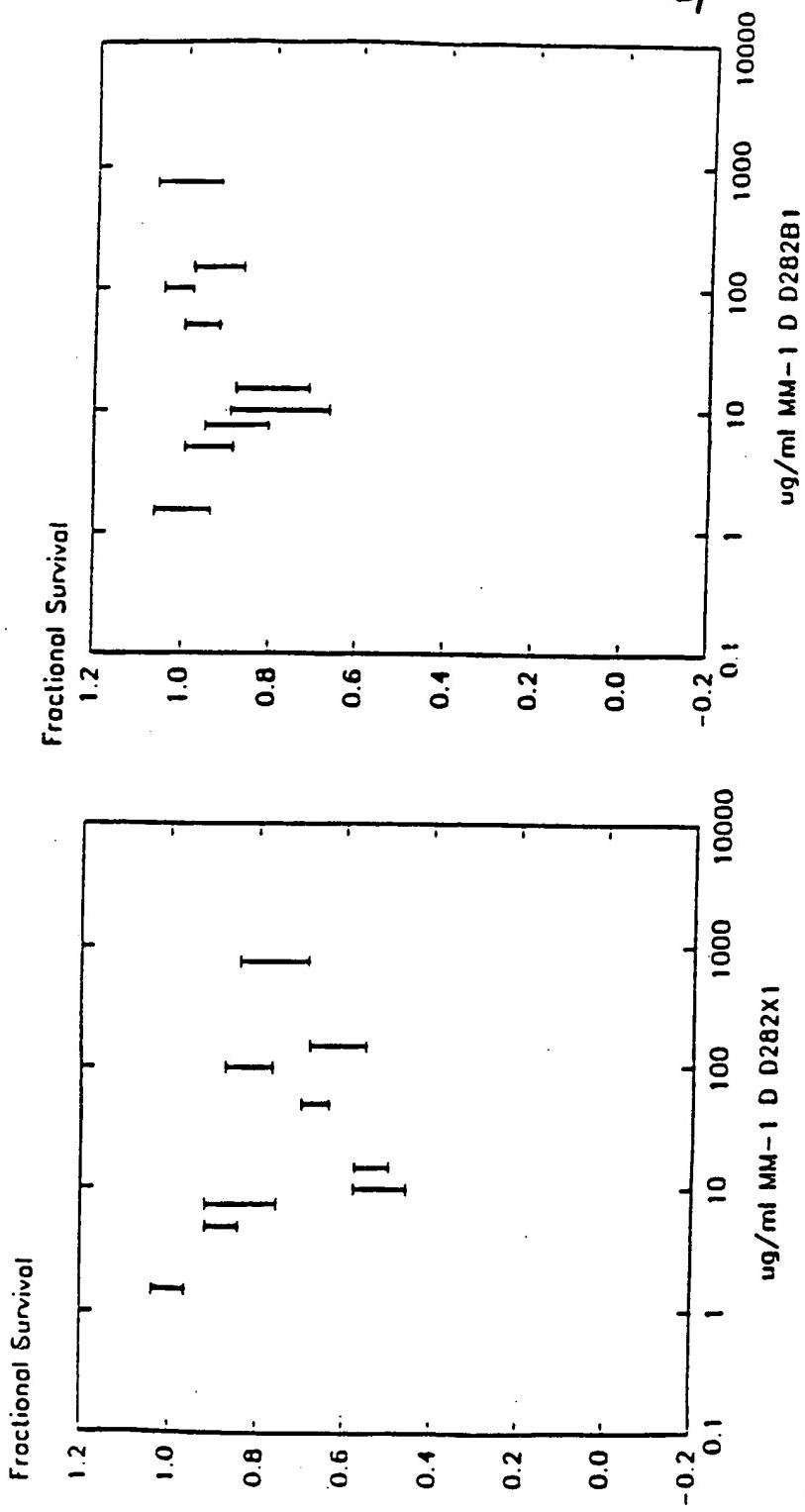
FIG. 17



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LANE 1 CONTAINS 0.5 µg OF MARKER (M) MONOMER-LENGTH KINETOPLAST DNA CIRCLES.
 LANES 2 AND 20 CONTAIN KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE
 PRESENCE OF 4% DMSO, BUT IN THE ABSENCE OF ANY COCOA PROCYANIDINS (CONTROL-C)
 LANES 3 AND 4 CONTAIN KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE
 PRESENCE OF 0.5 AND 5.0 µg/ml COCOA PROCYANIDIN FRACTION A.
 LANES 5 AND 6 CONTAIN KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE
 PRESENCE OF 0.5 AND 5.0 µg/ml COCOA PROCYANIDIN FRACTION B.
 LANES 7,8,9,13,14 AND 15 ARE REPLICATES OF KINETOPLAST DNA THAT WAS INCUBATED WITH
 TOPOISOMERASE II IN THE PRESENCE OF 0.05, 0.5 AND 5.0 µg/ml COCOA PROCYANIDIN FRACTION D.
 LANES 10,11,12,16,17 AND 18 ARE REPLICATES OF KINETOPLAST DNA THAT WAS INCUBATED WITH
 TOPOISOMERASE II IN THE PRESENCE OF 0.05, 0.5 AND 5.0 µg/ml COCOA PROCYANIDIN FRACTION E.
 LANE 19 IS A REPLICATE OF KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE
 PRESENCE OF 5.0 µg/ml COCOA PROCYANIDIN FRACTION E.

SUBSTITUTE SHEET (RULE 26)

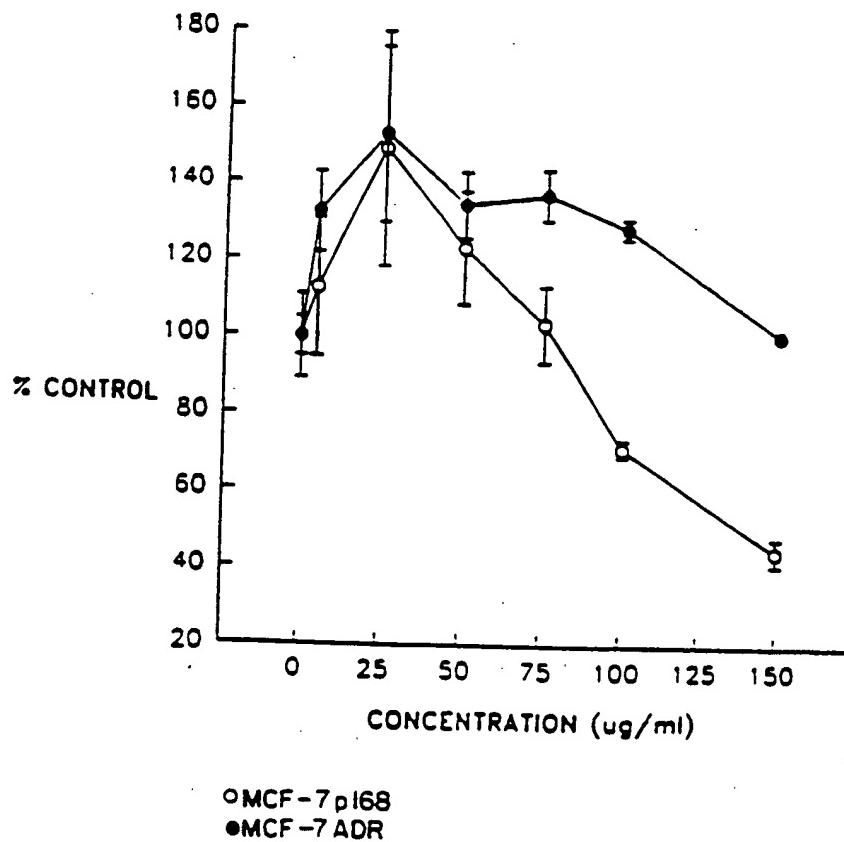
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SUBSTITUTE SHEET (RULE 26)

*FIG. 18A**FIG. 18B*

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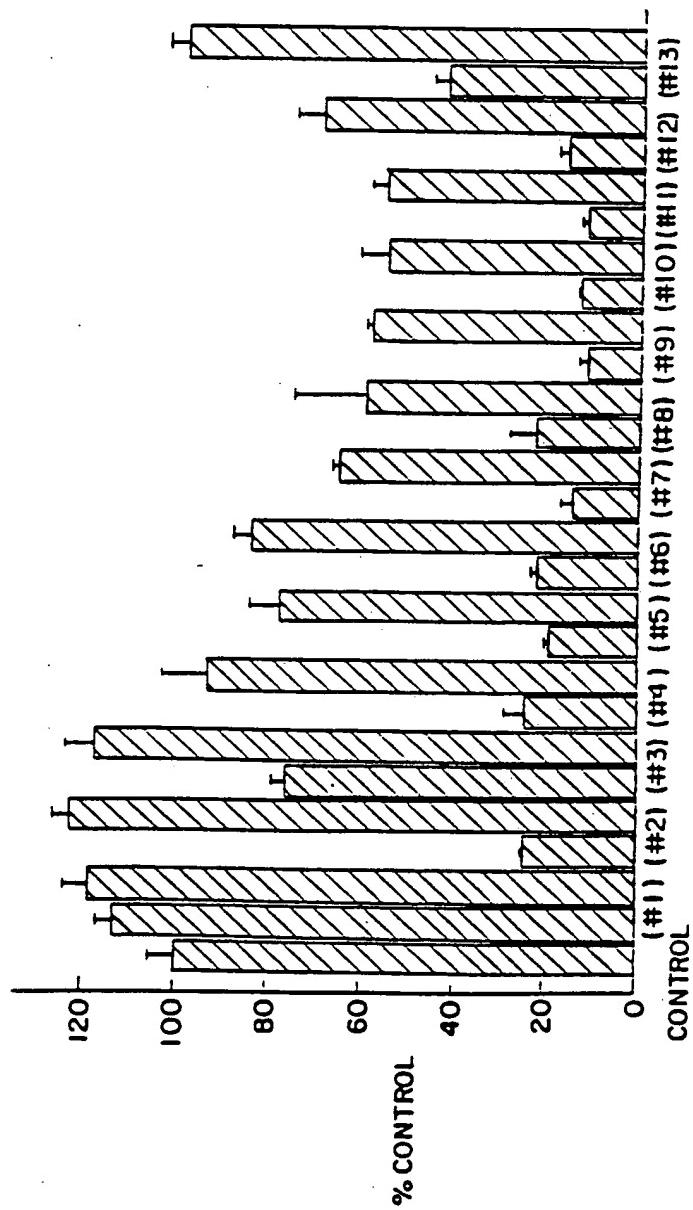
FIG. 19



SUBSTITUTE SHEET (RULE 26)

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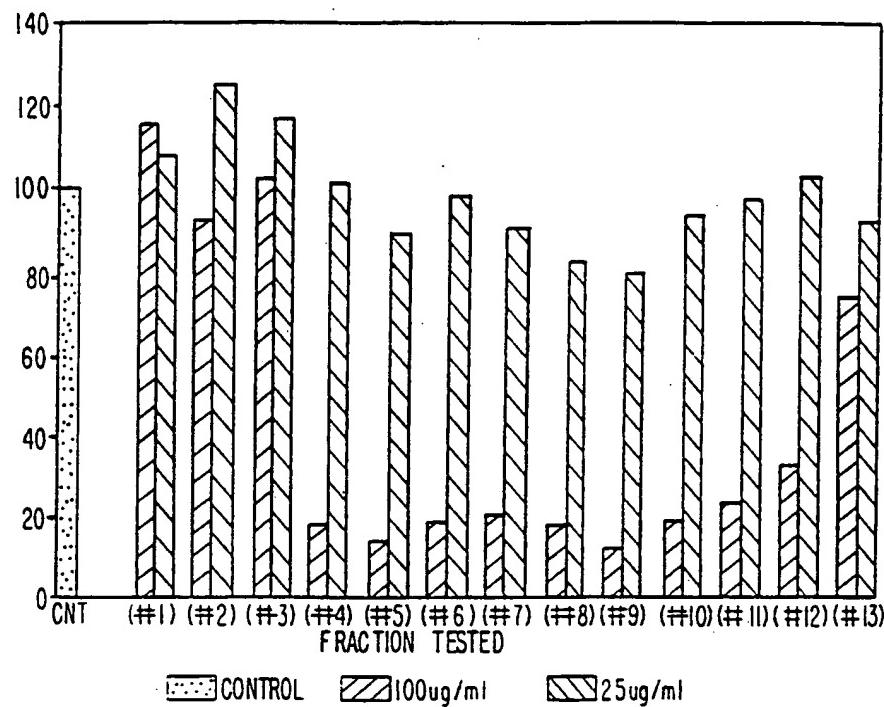
FIG. 20A



SUBSTITUTE SHEET (RULE 26)

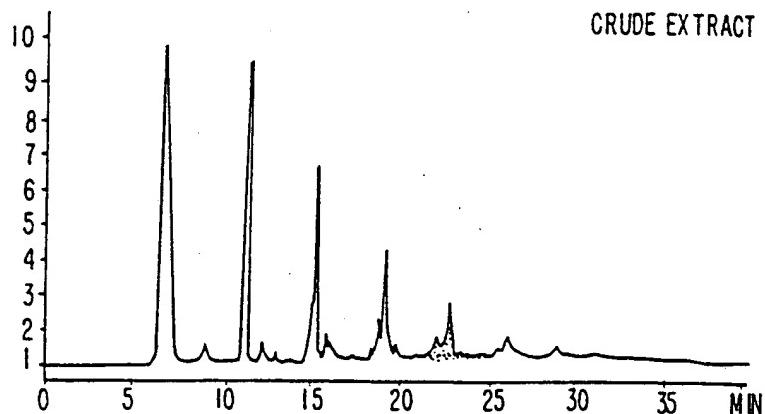
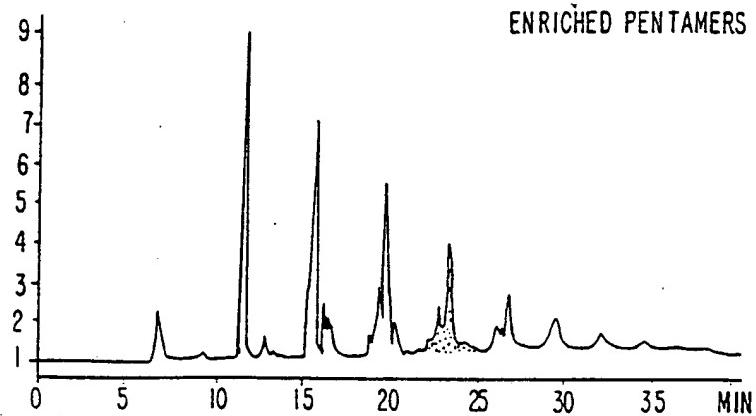
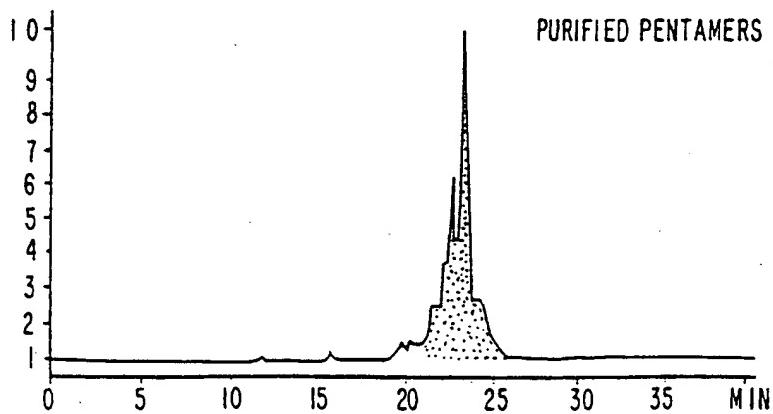
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FIG. 20B



FRACTION NUMBER OLIGOMERIC UNIT

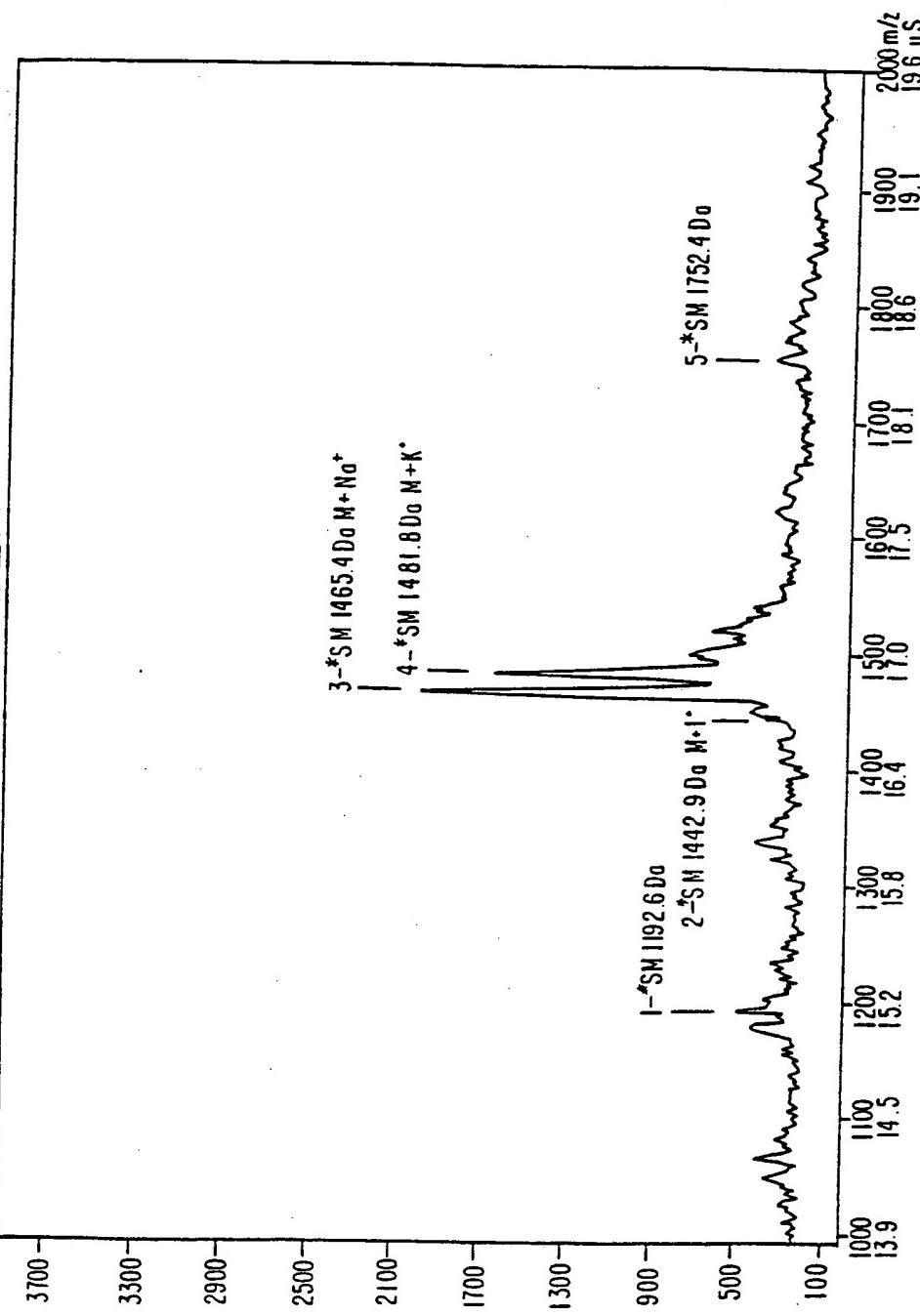
#1	DIMERS
#2	TRIMERS
#3	TETRAMERS
#4	PENTAMERS
#5	HEXAMERS
#6	HEPTAMERS
#7	OCTAMERS
#8	NONAMERS
#9	DECAMERS
#10	UNDECAMERS
#11	DODECAMERS
#12	HIGHER OLIGOMERS
#13	UNKNOWN

FIG. 21A**FIG. 21B****FIG. 21C**

SUBSTITUTE SHEET (RULE 26)

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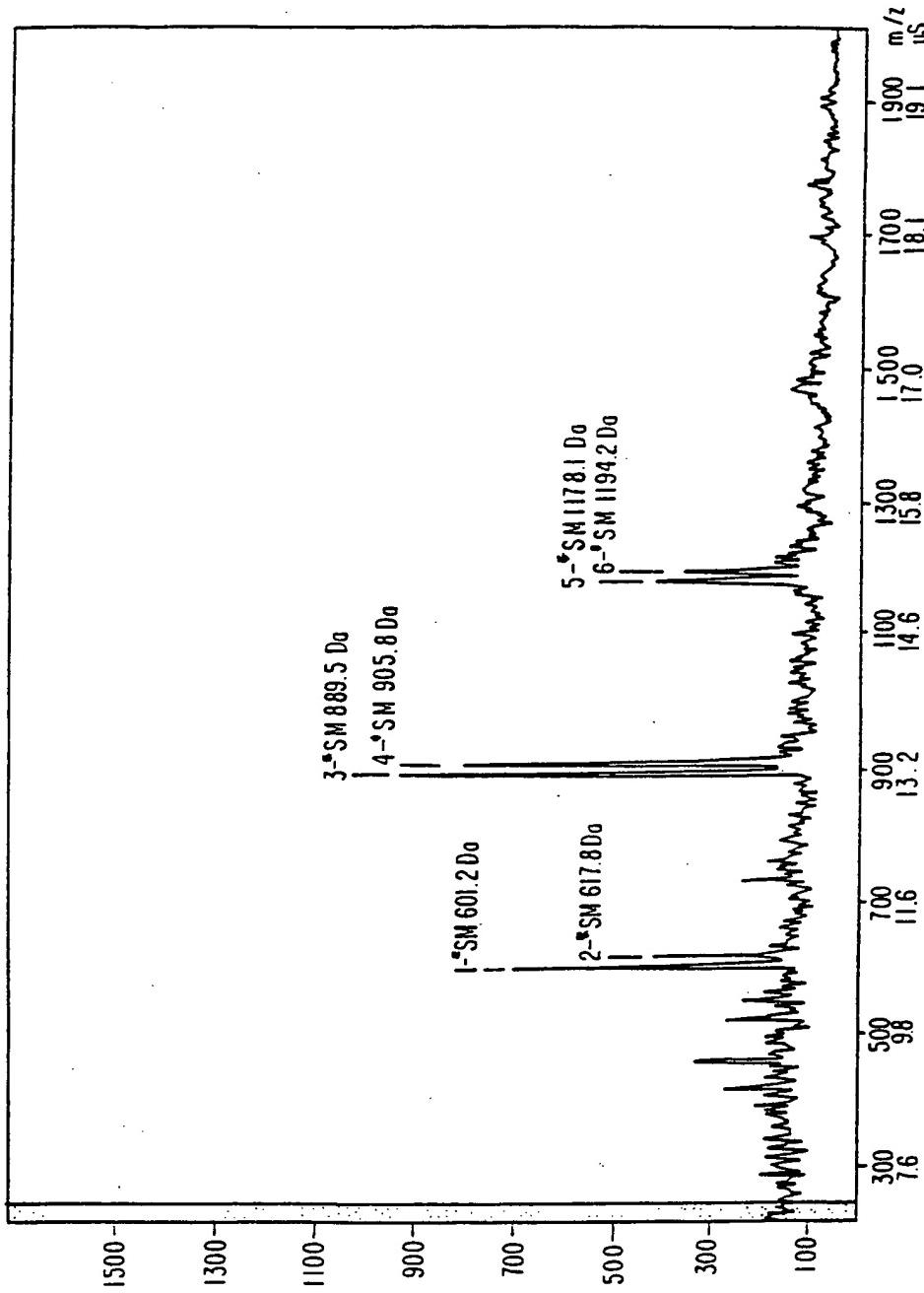
FIG. 22A



SUBSTITUTE SHEET (RULE 26)

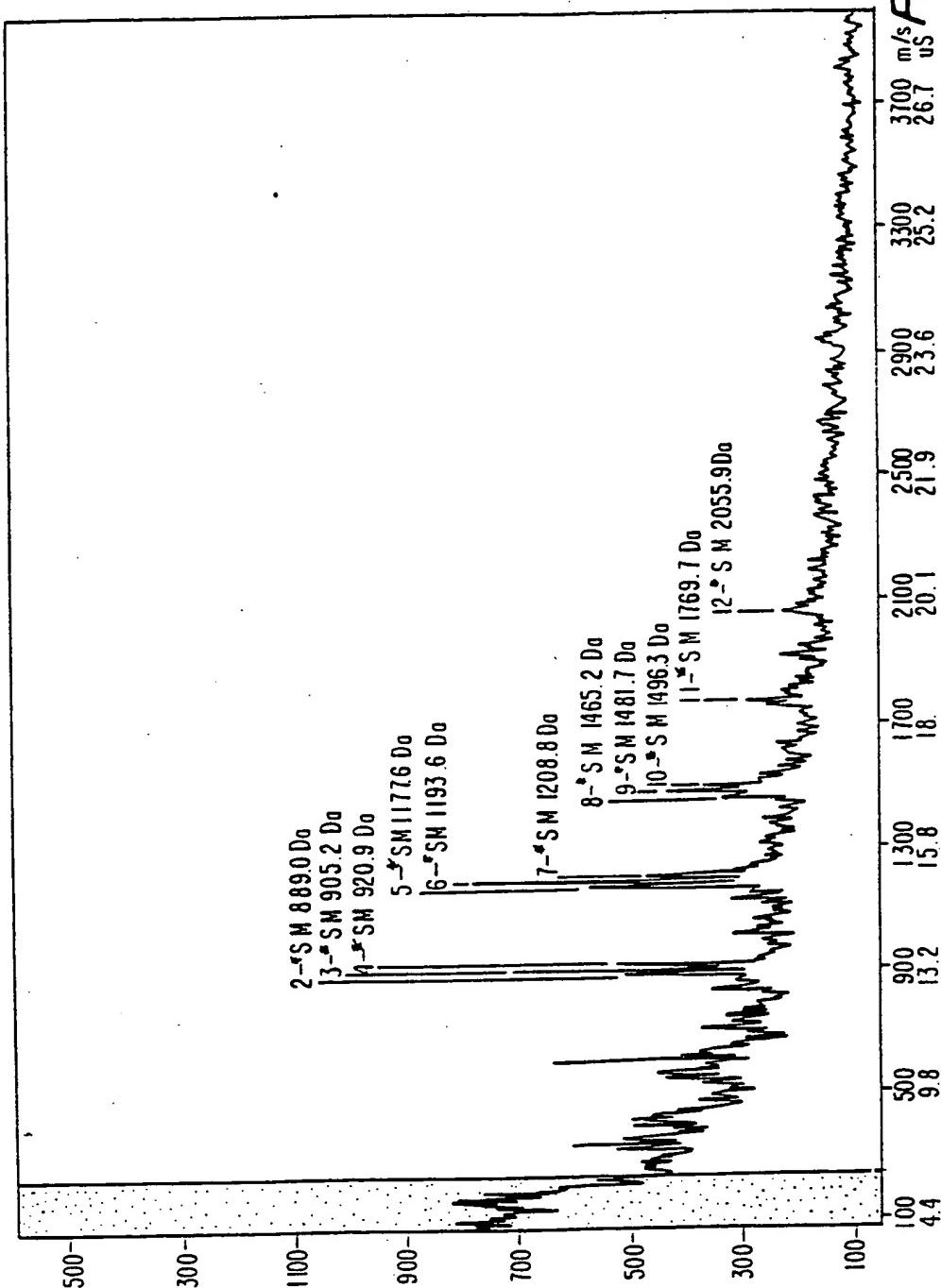
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FIG. 22B



SUBSTITUTE SHEET (RULE 26)

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SUBSTITUTE SHEET (RULE 26)

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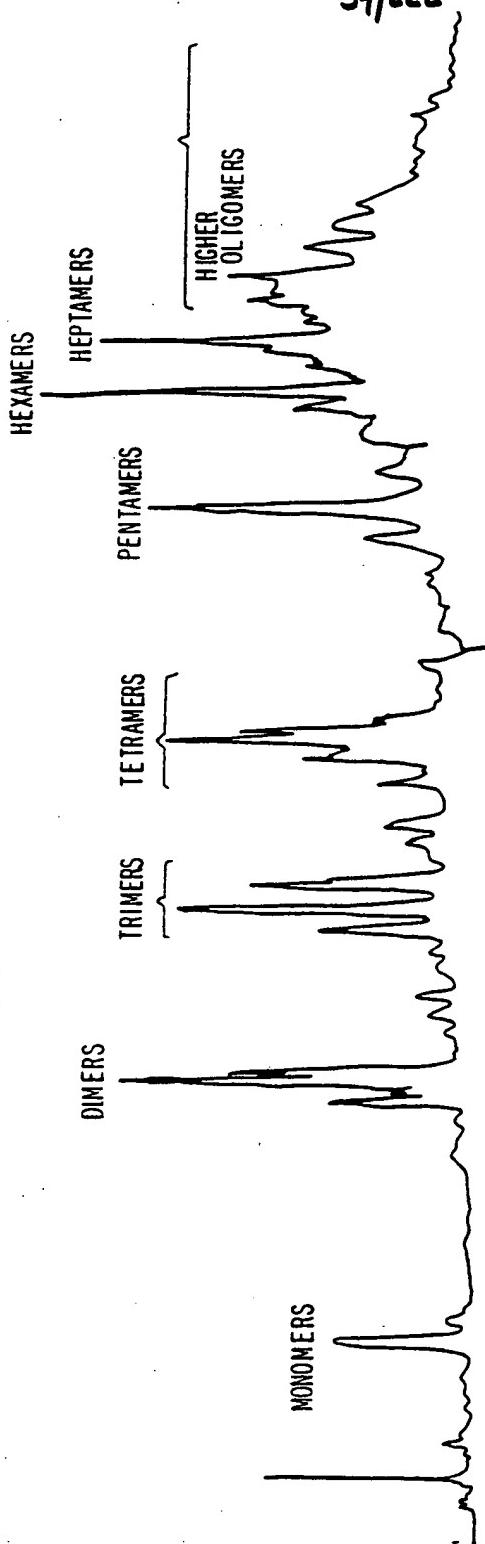
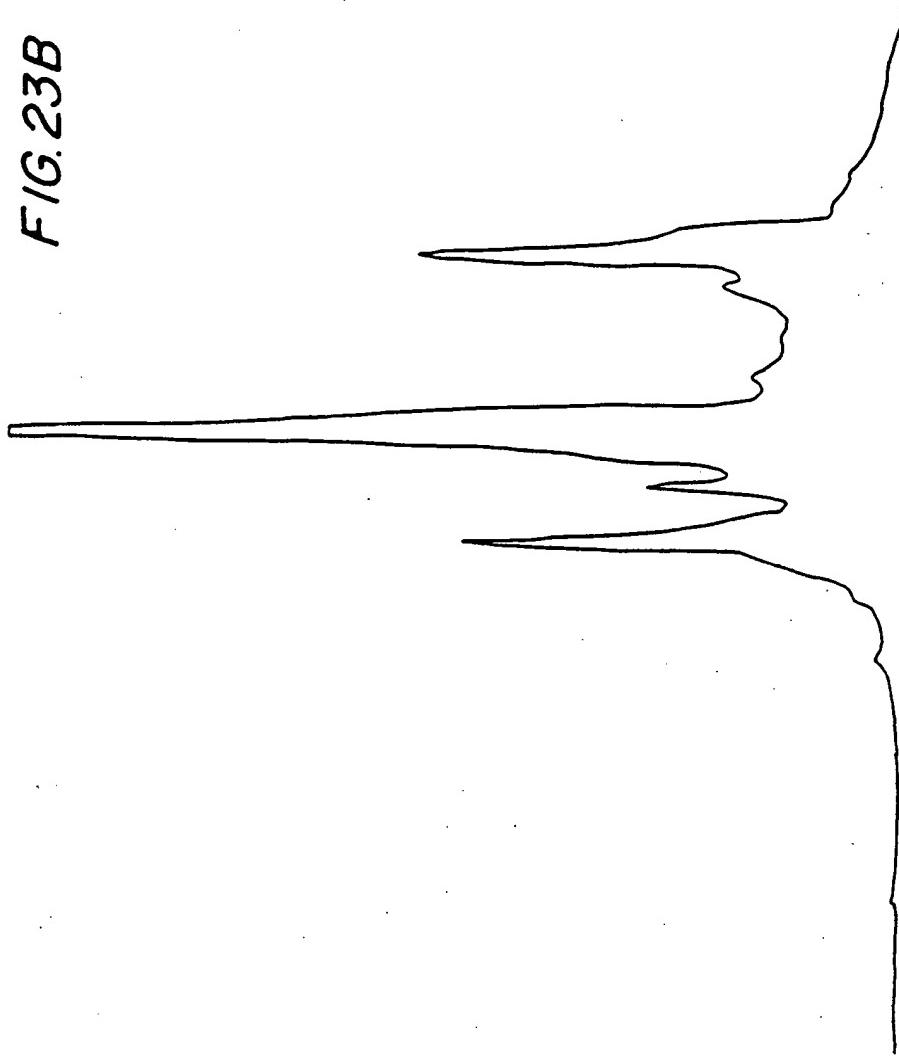


FIG. 23A

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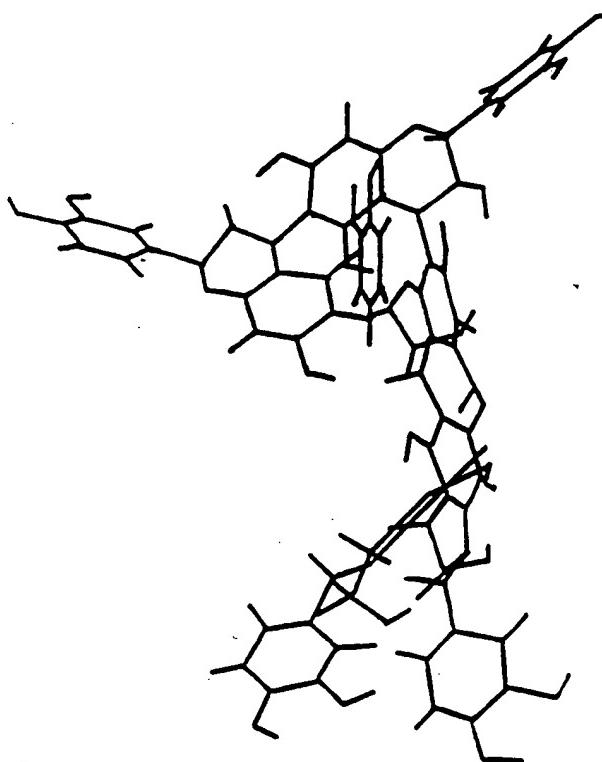
FIG. 23B



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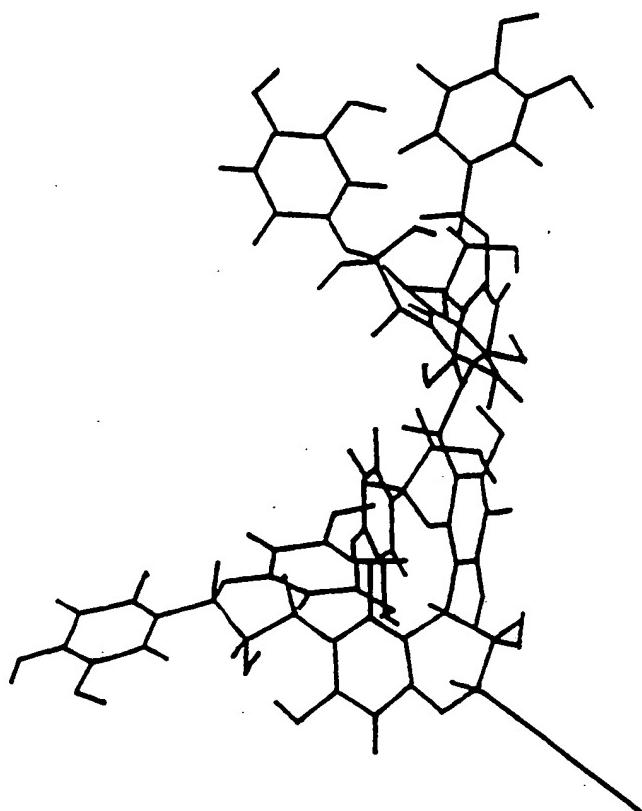
FIG. 24A



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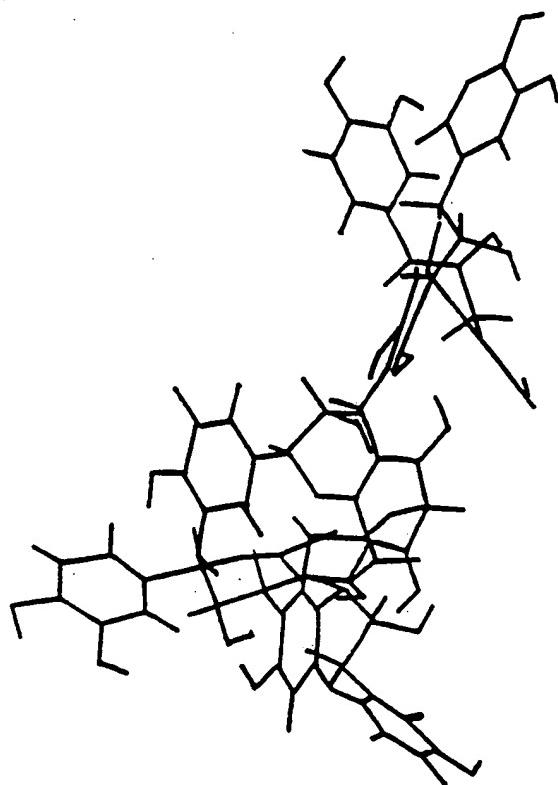
FIG. 24B



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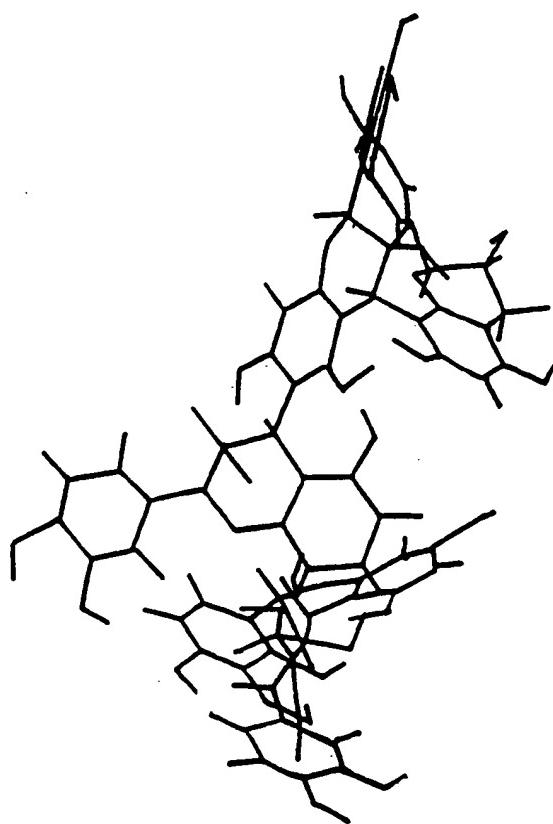
FIG. 24C



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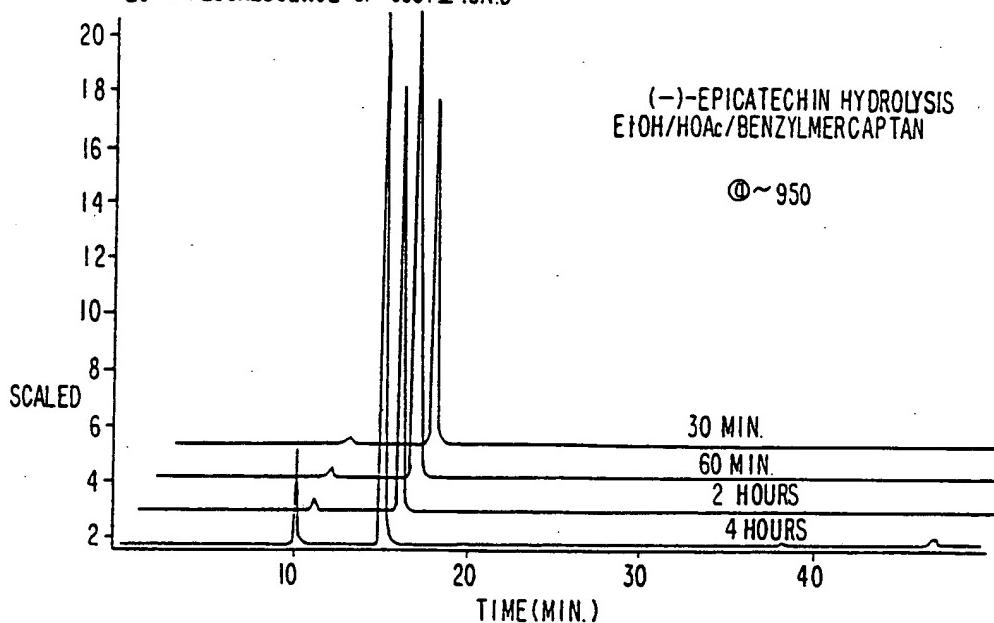
FIG. 24D



SUBSTITUTE SHEET (RULE 26)

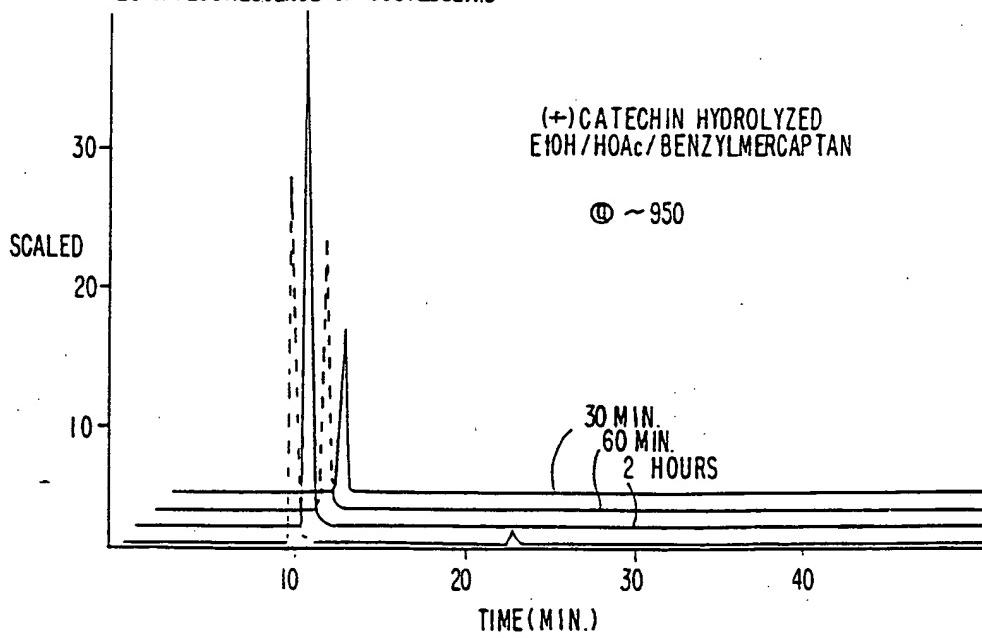
LC X FLUORESCENCE OF 6057_45A.D
 LC X FLUORESCENCE OF 6057_46A.D
 LC X FLUORESCENCE OF 6057_47A.D
 LC X FLUORESCENCE OF 6057_48A.D

FIG. 25A



LC X FLUORESCENCE OF 6057_49A.D
 LC X FLUORESCENCE OF 6057_50A.D
 LC X FLUORESCENCE OF 6057_51A.D
 LC X FLUORESCENCE OF 6057_52A.D

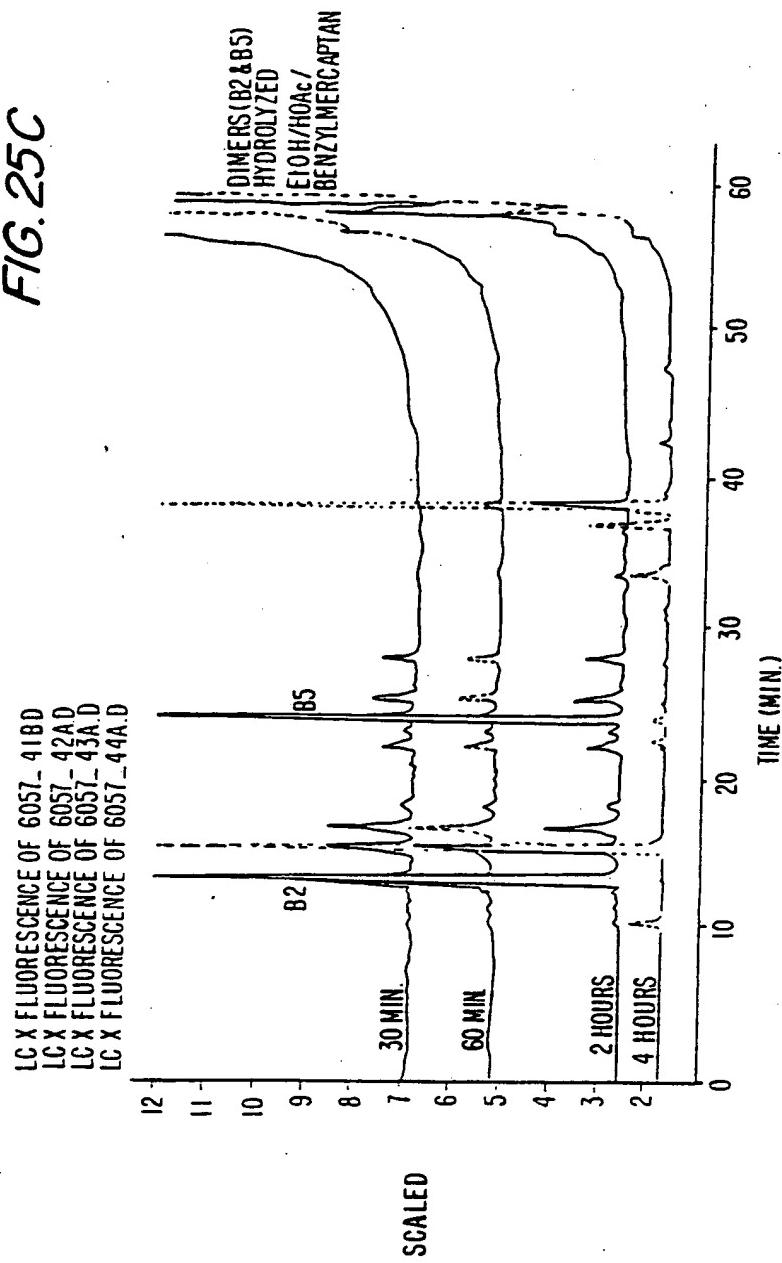
FIG. 25B



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FIG. 25C



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FIG. 26A

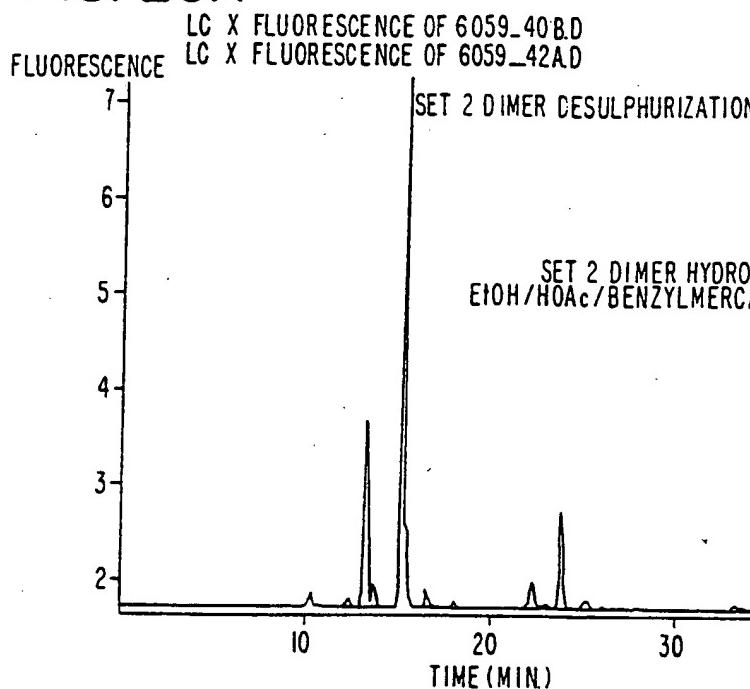
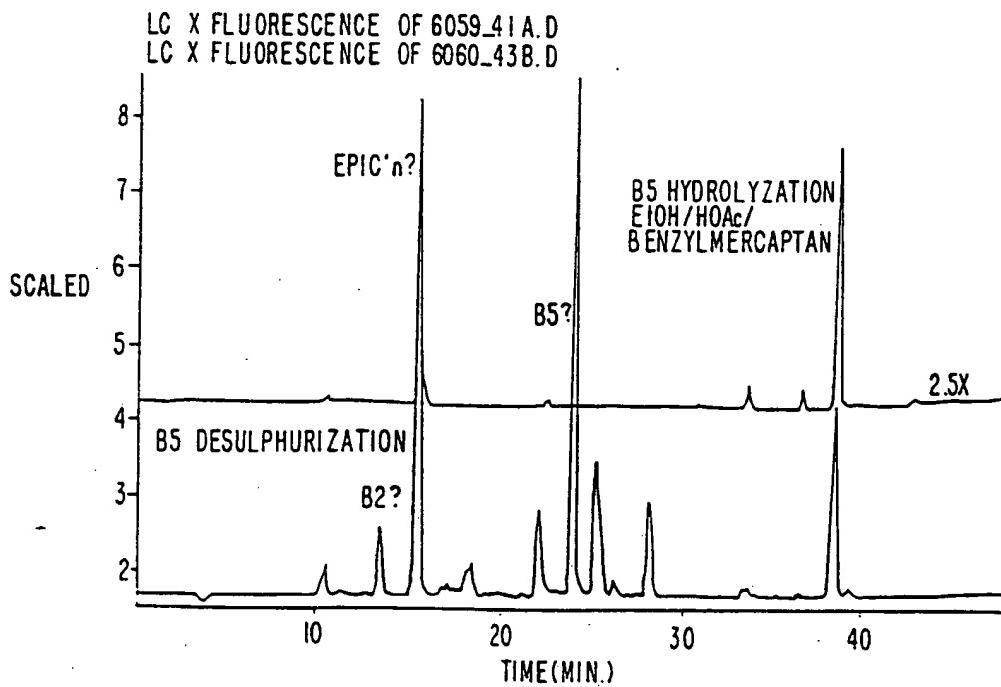


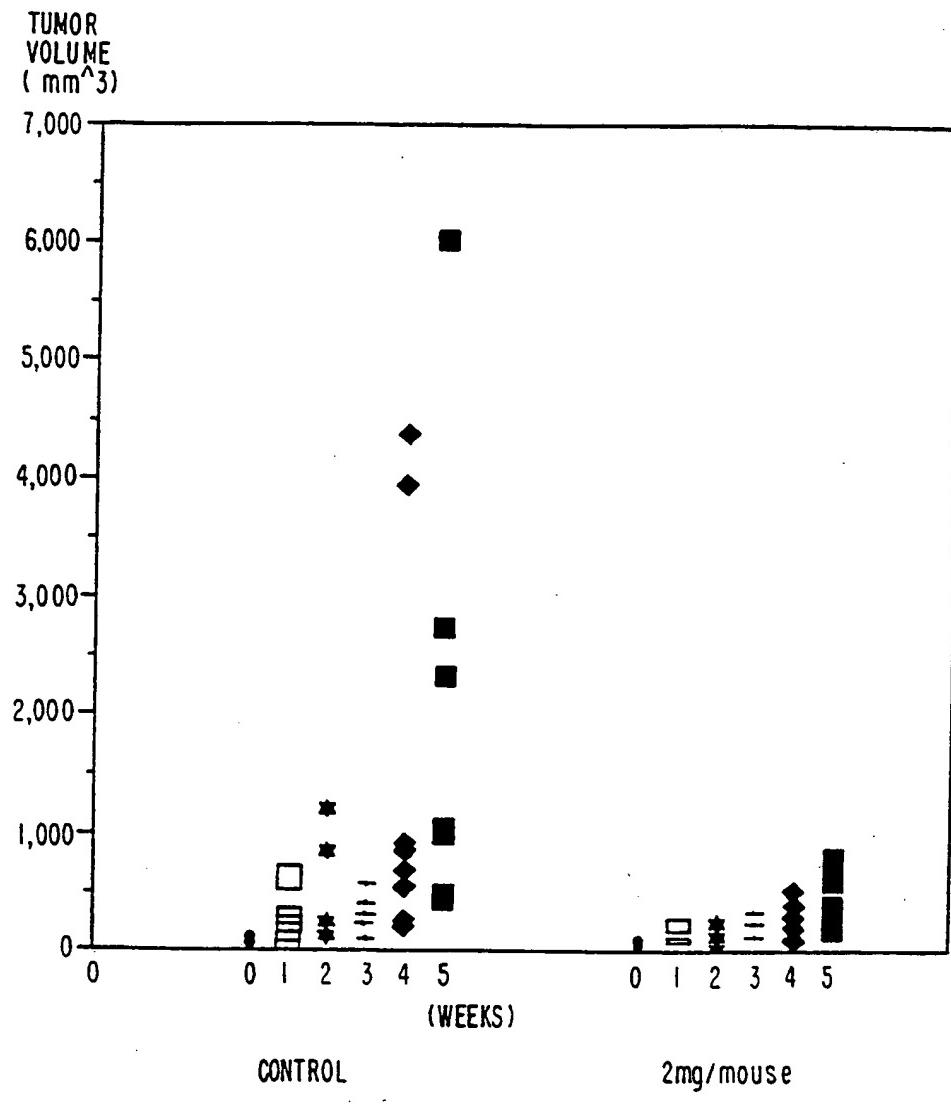
FIG. 26B



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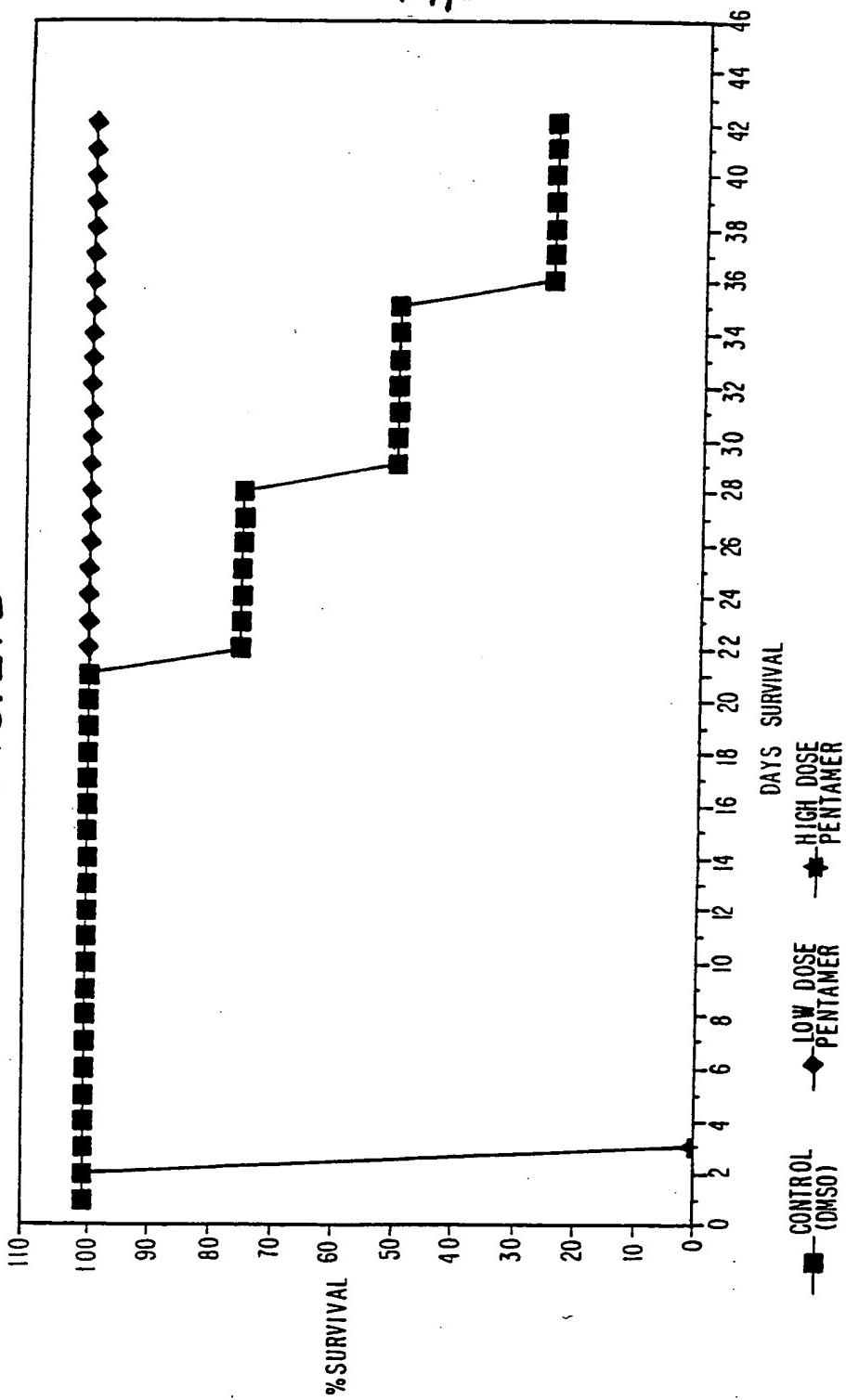
FIG. 27A



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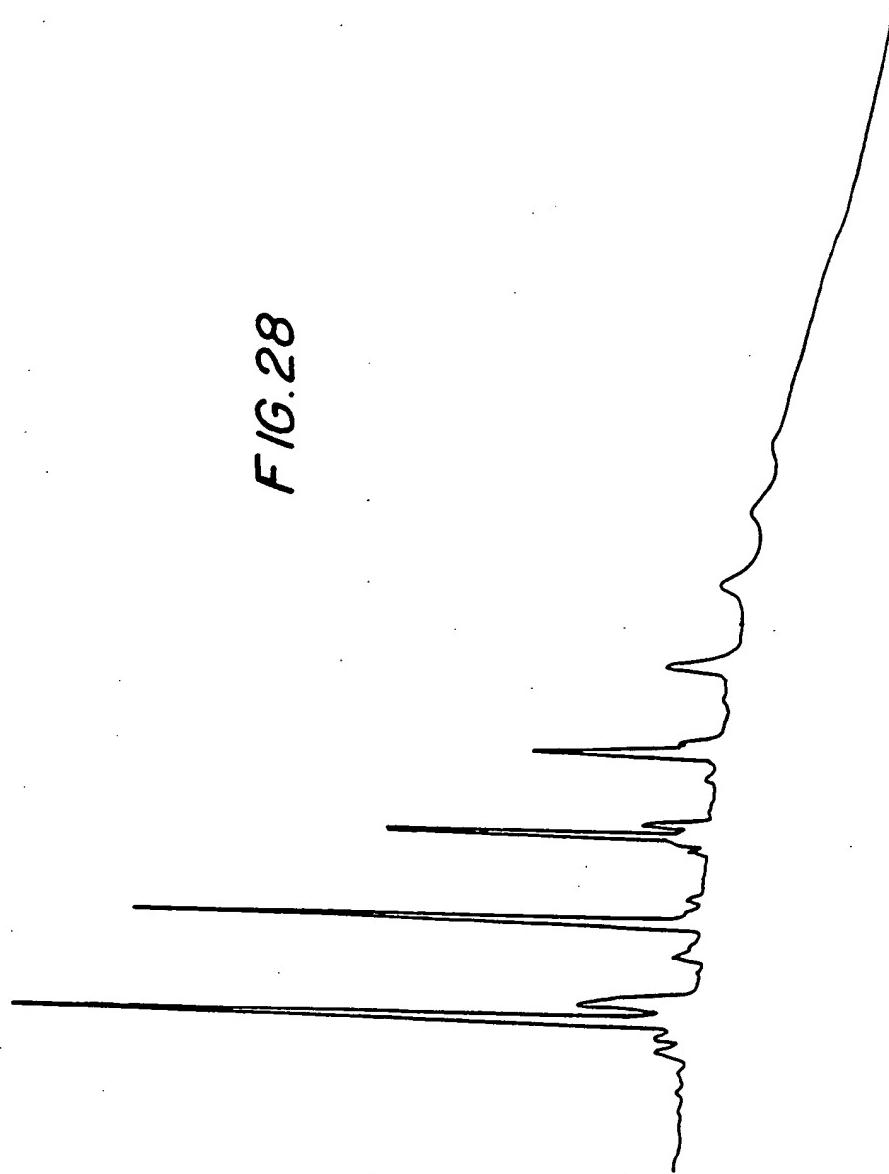
FIG. 27B



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FIG. 28



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FIG. 29A

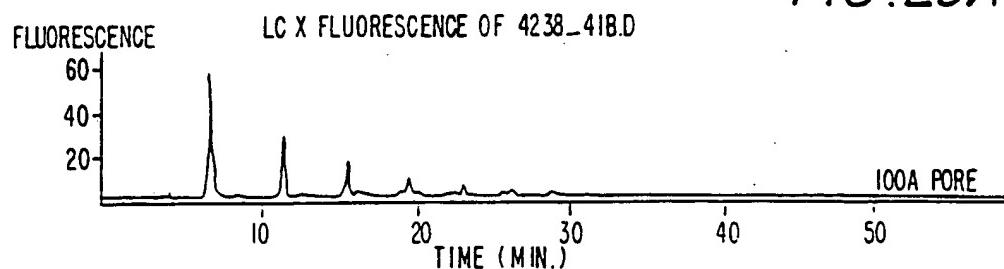


FIG. 29B

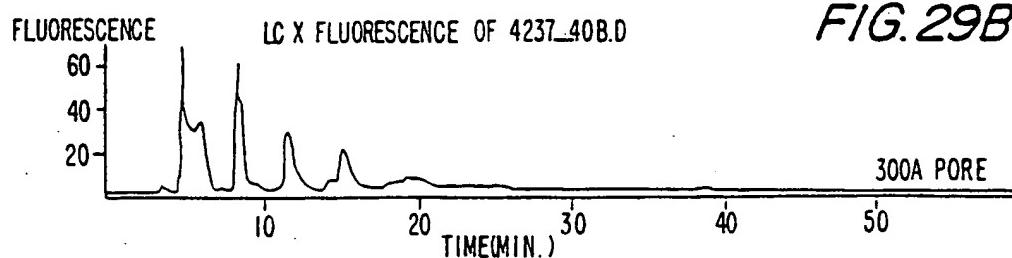


FIG. 29C

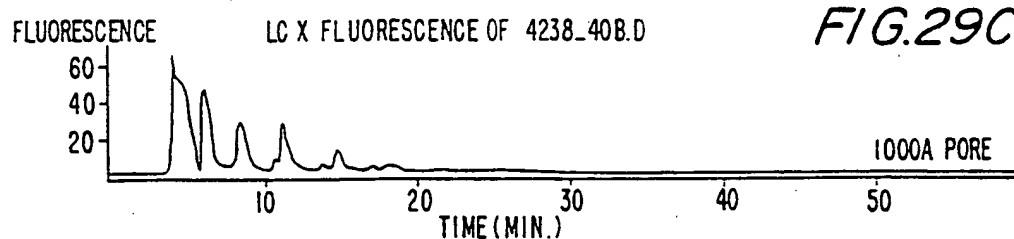


FIG.30A

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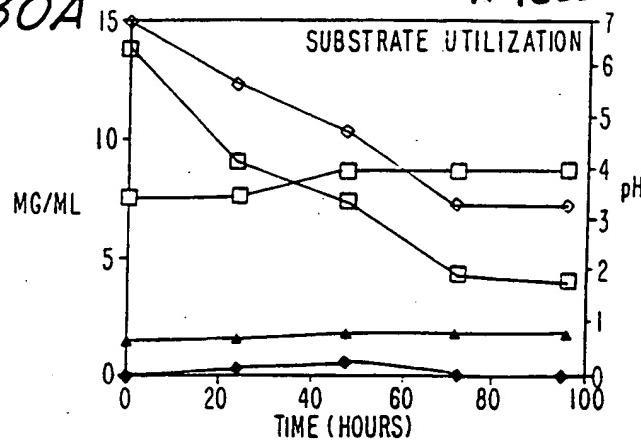


FIG.30B

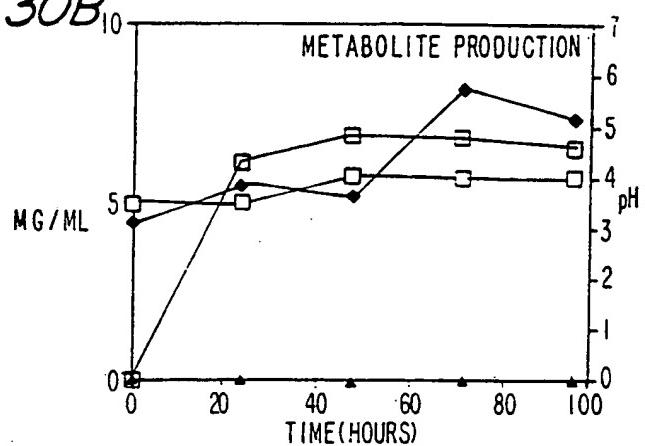
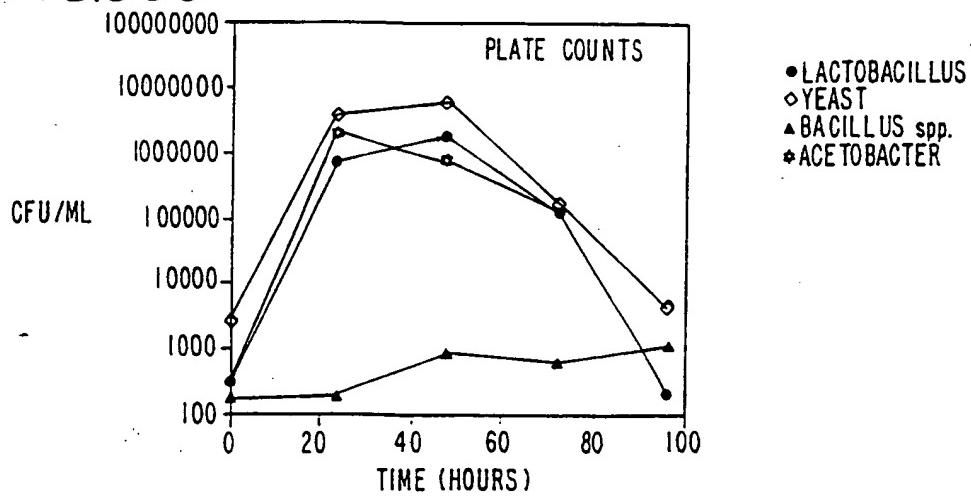


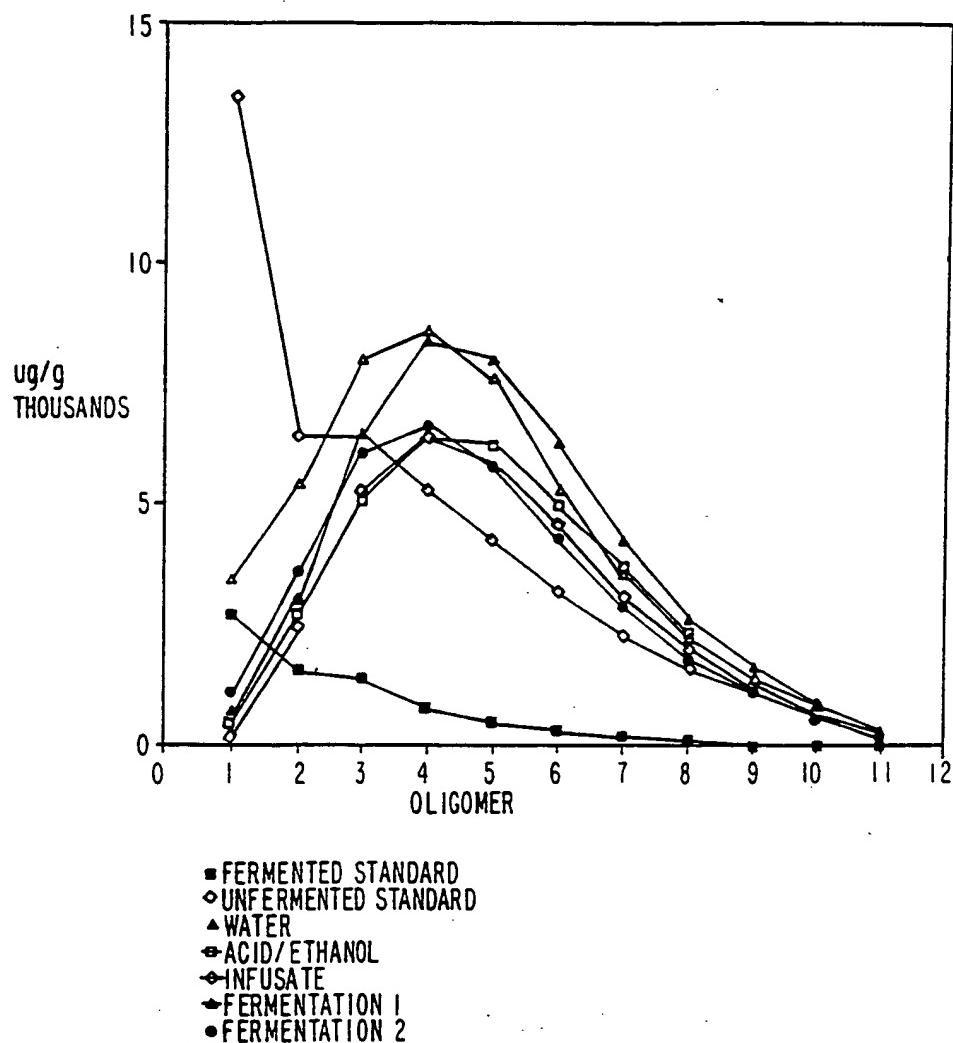
FIG.30C



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FIG. 30D

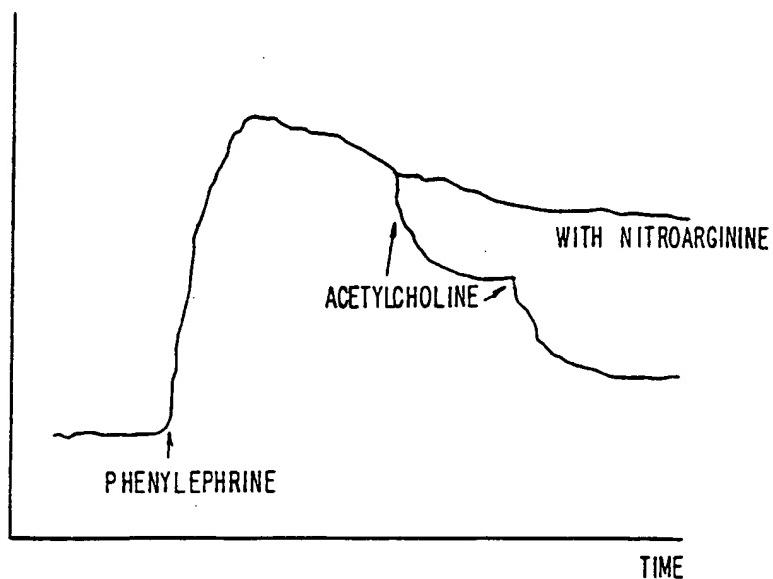


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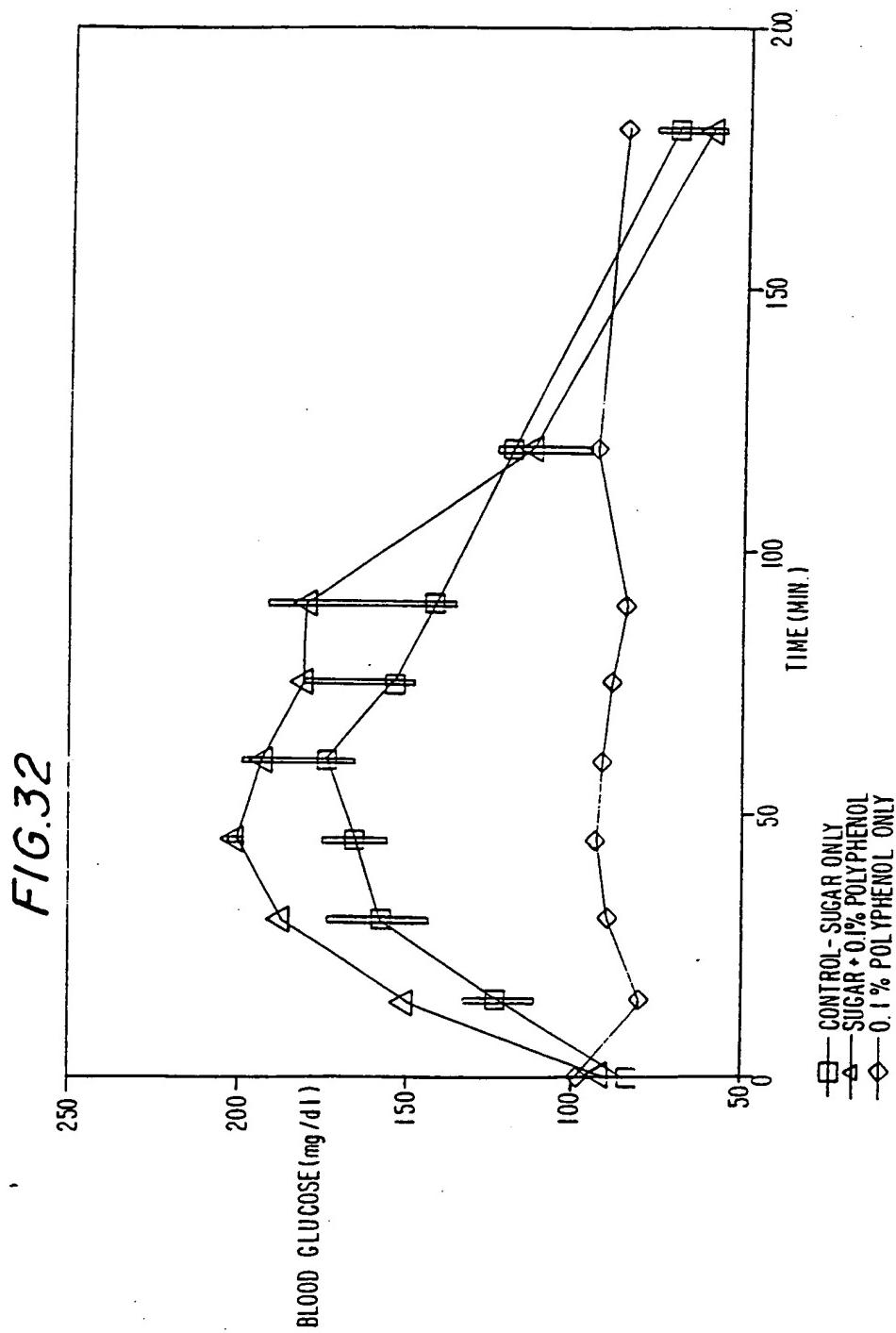
FIG.31

CONTRACTION OF ISOLATED AORTA



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FIG. 33A

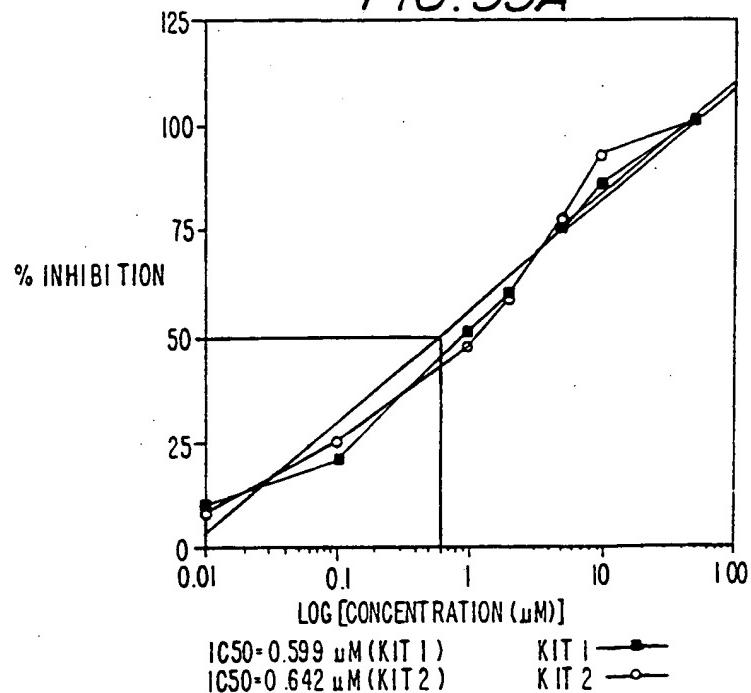
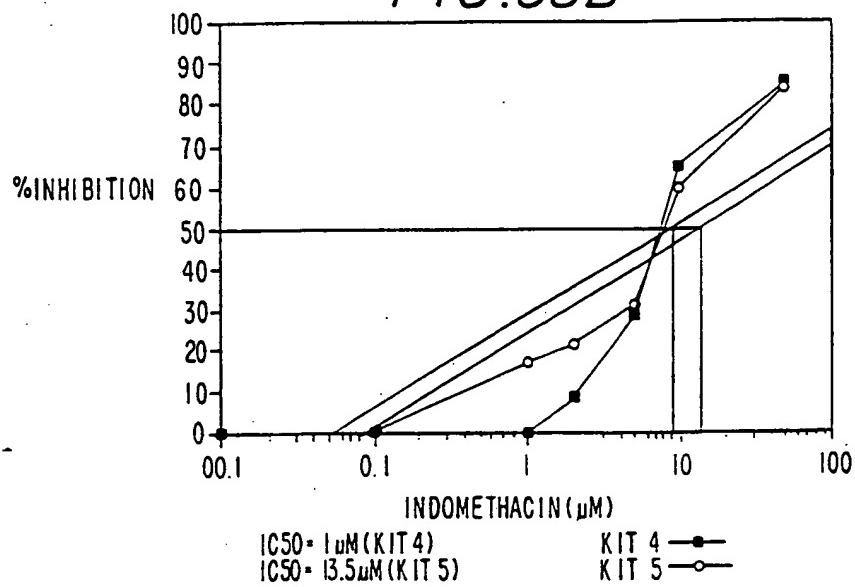


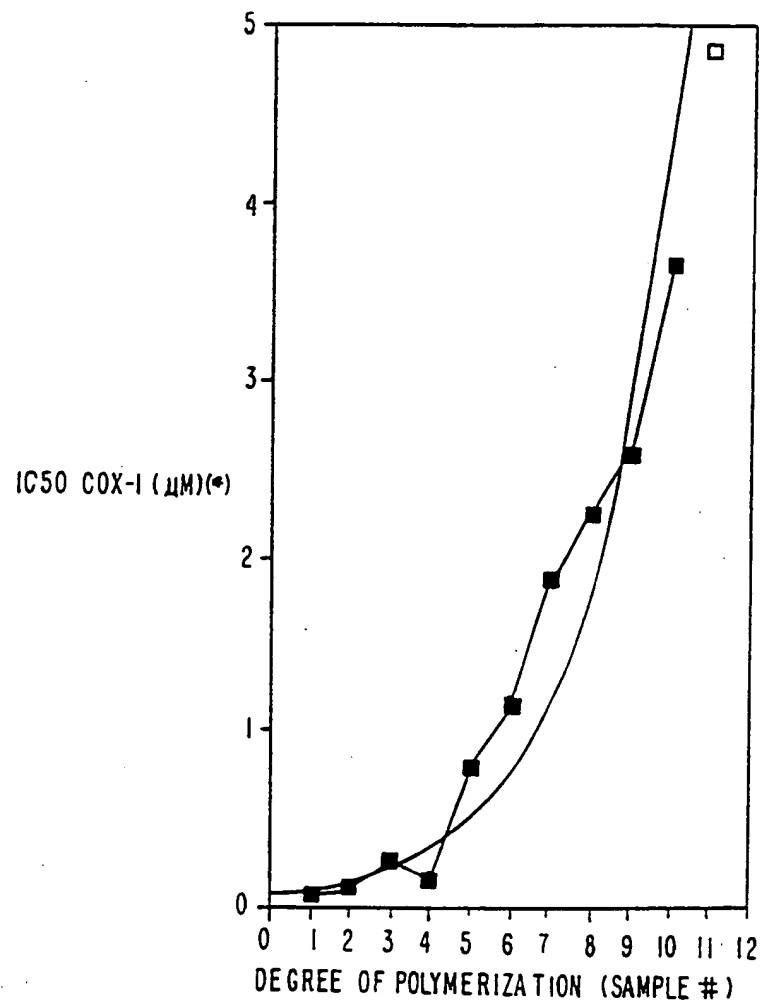
FIG. 33B



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FIG. 34A

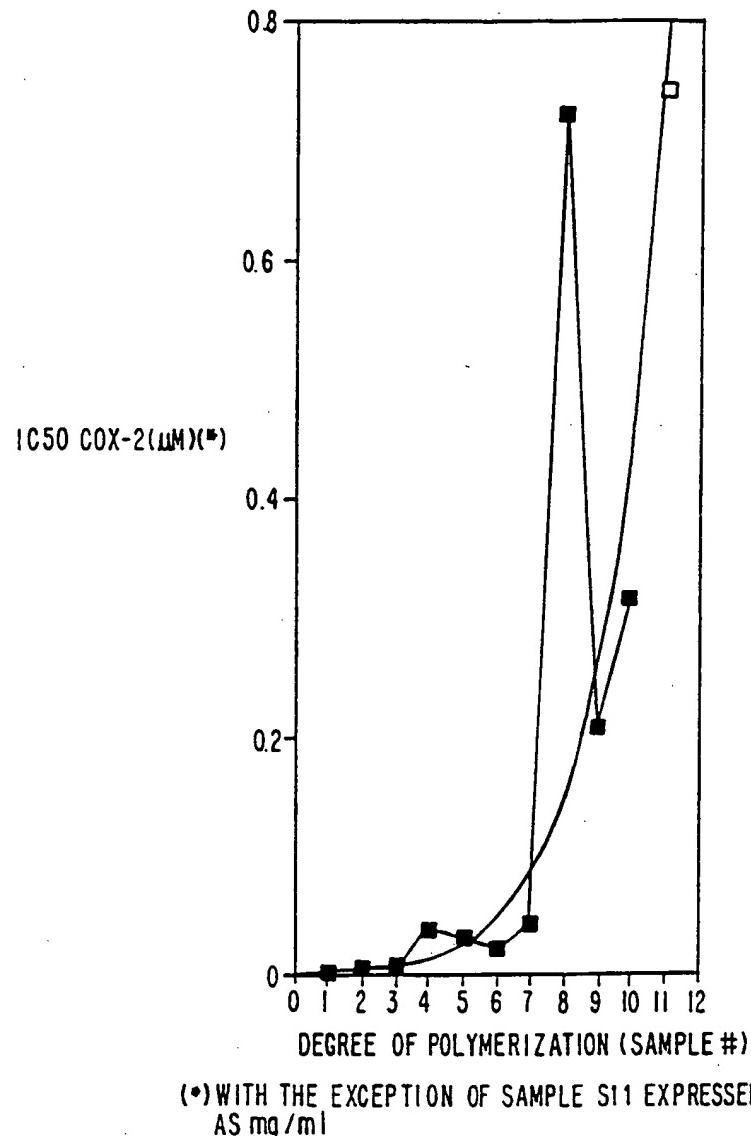


(•) WITH THE EXCEPTION OF SAMPLE S11 EXPRESSED AS mg/ml

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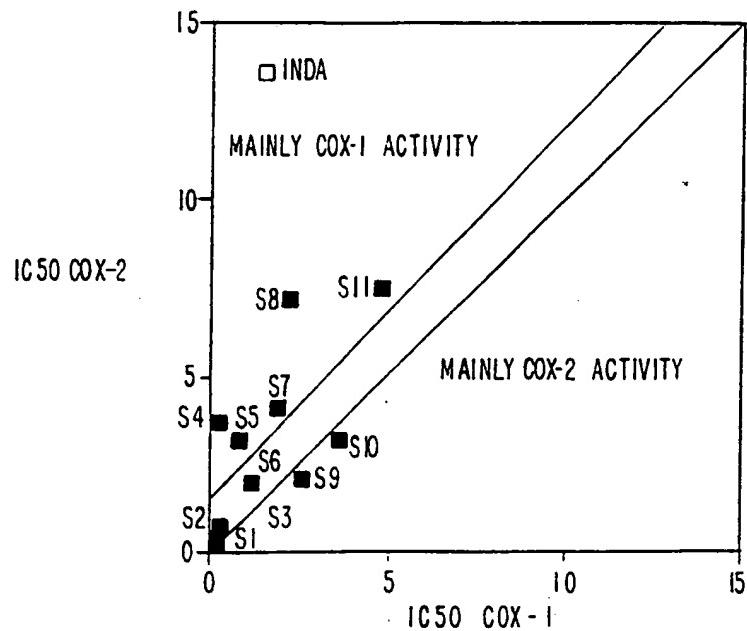
FIG. 34B



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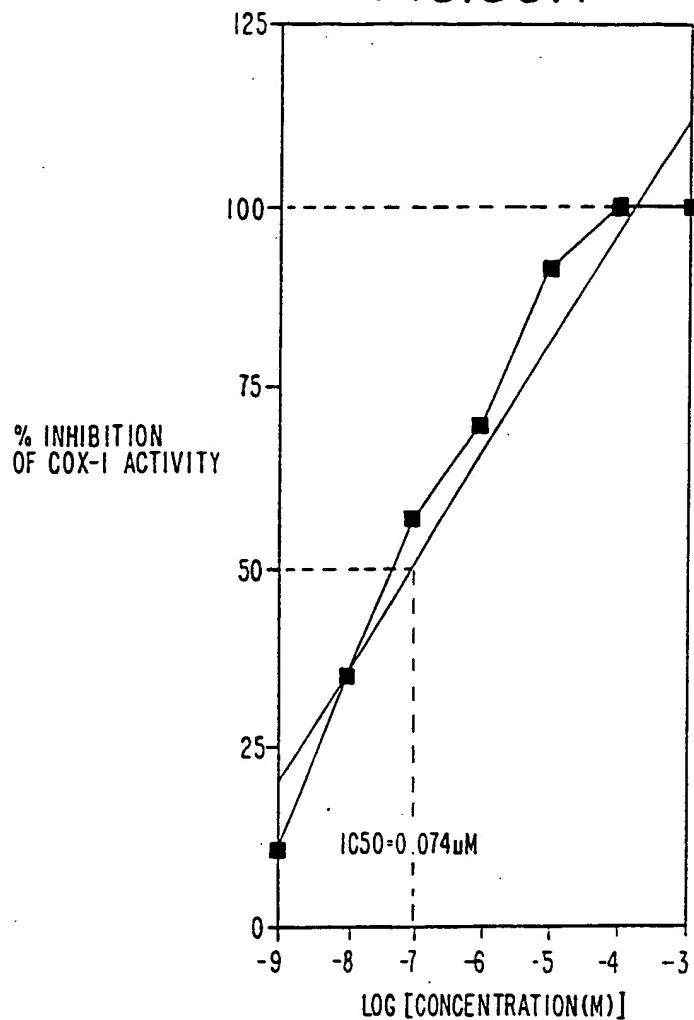
FIG. 35



(*) WITH THE EXCEPTION OF SAMPLE S11

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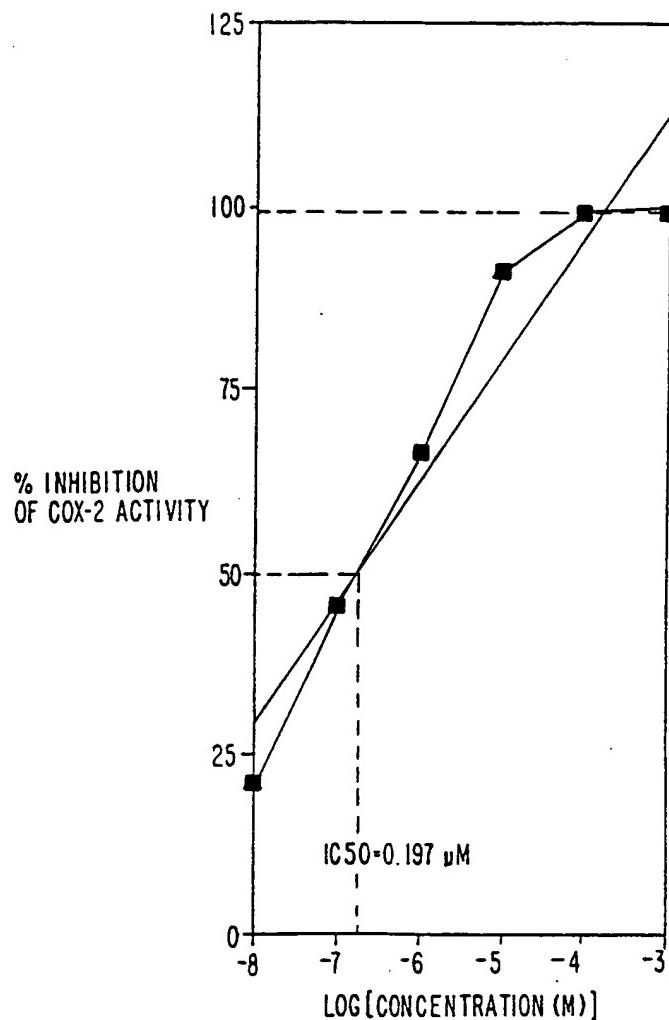
FIG. 36A



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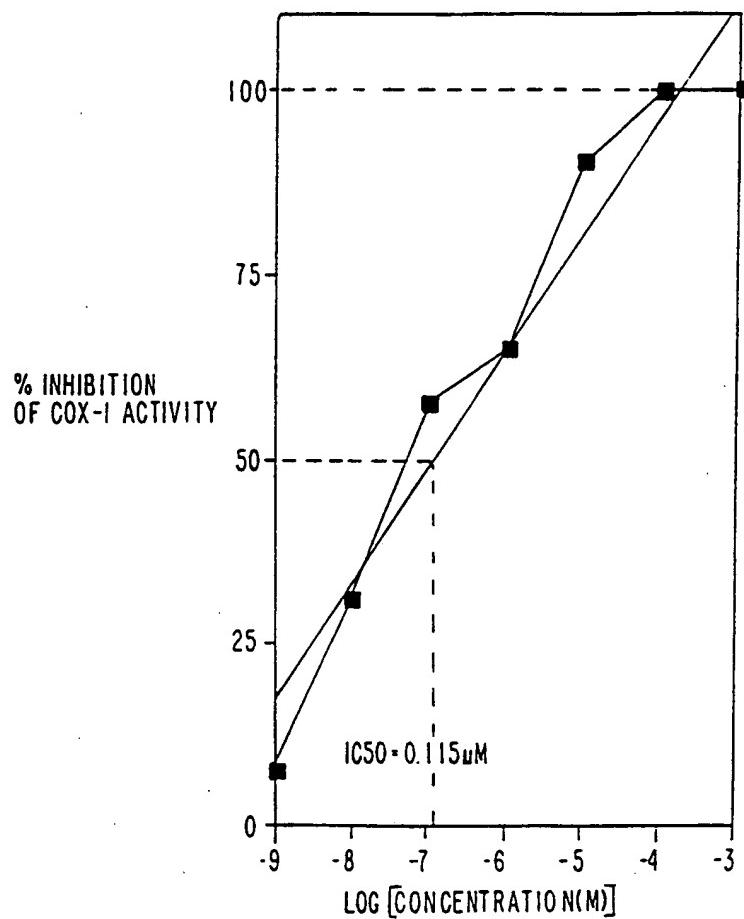
FIG. 36B



SUBSTITUTE SHEET (RULE 26)

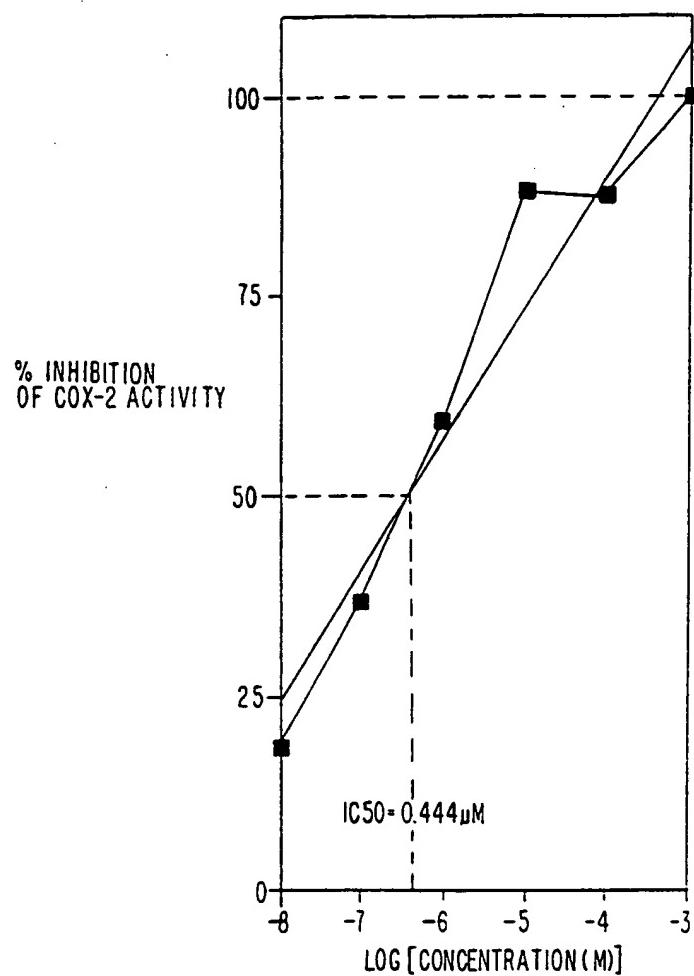
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FIG. 36C



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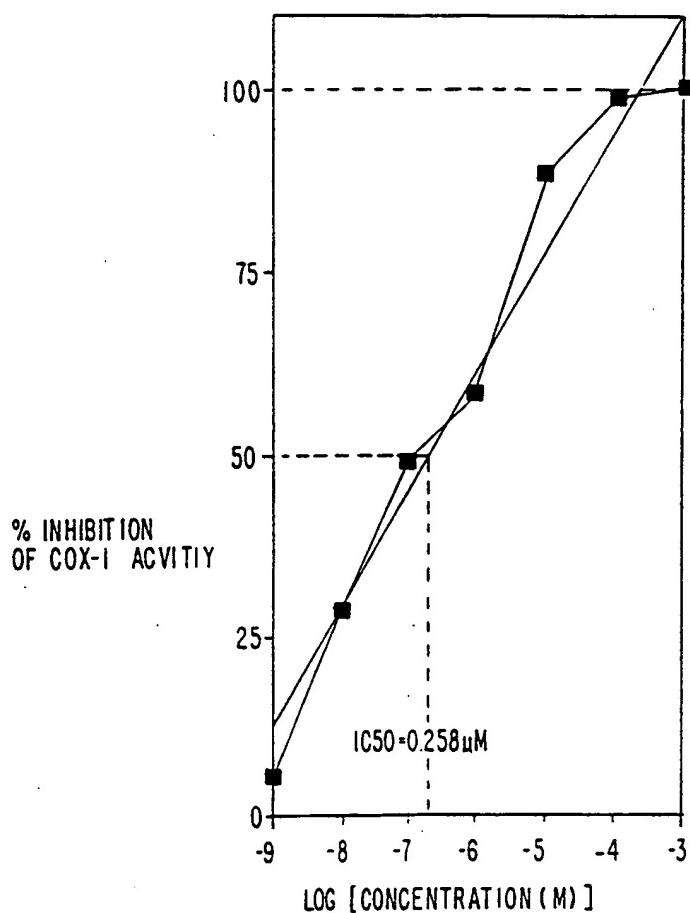
FIG. 36D



SUBSTITUTE SHEET (RULE 26)

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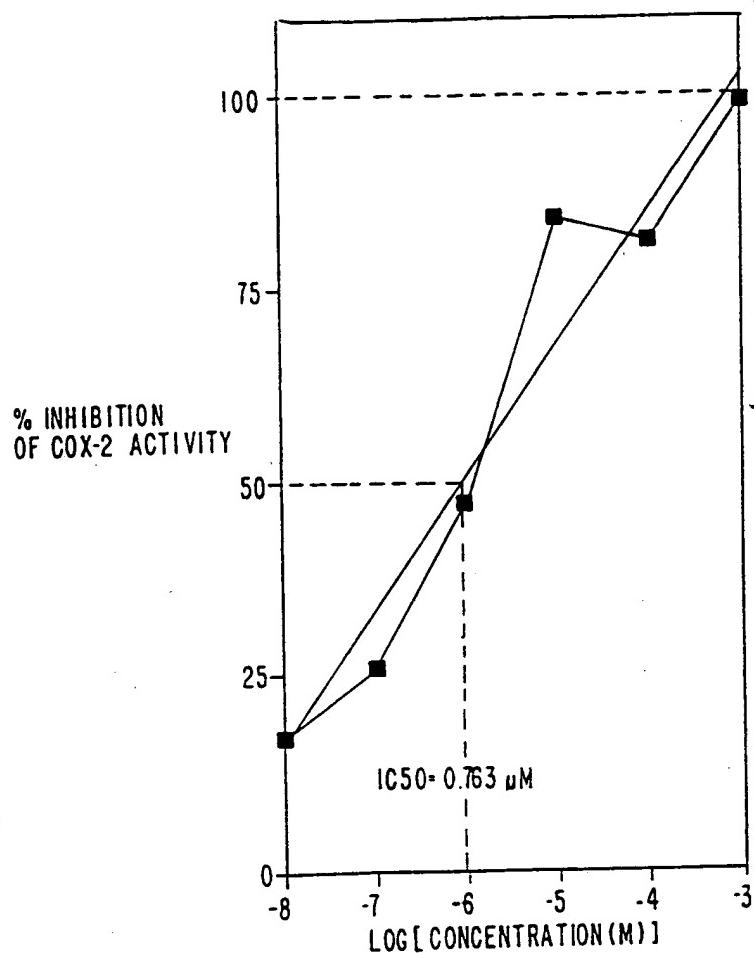
FIG.36E



SUBSTITUTE SHEET (RULE 26)

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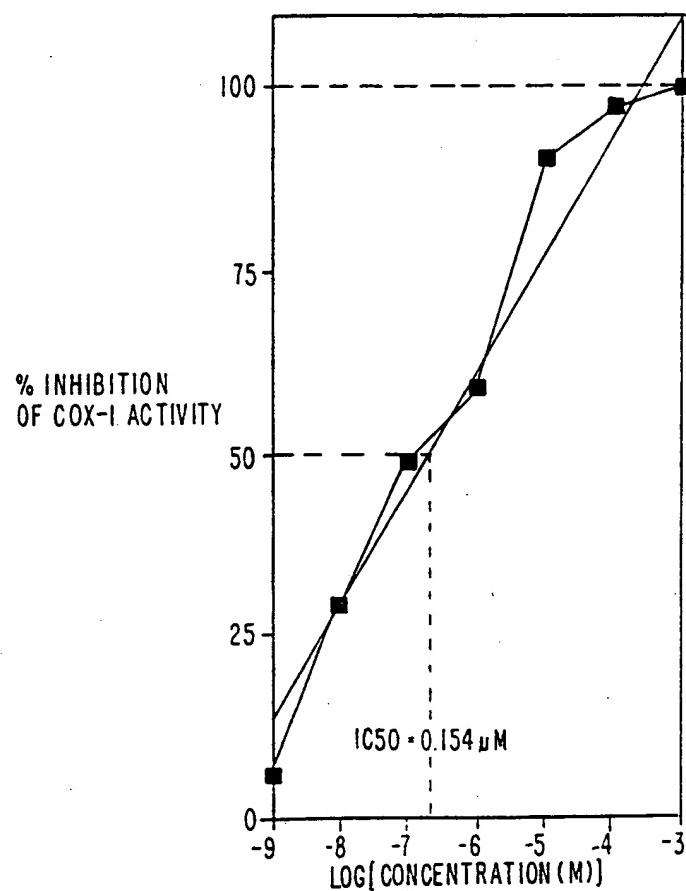
FIG. 36F



SUBSTITUTE SHEET (RULE 26)

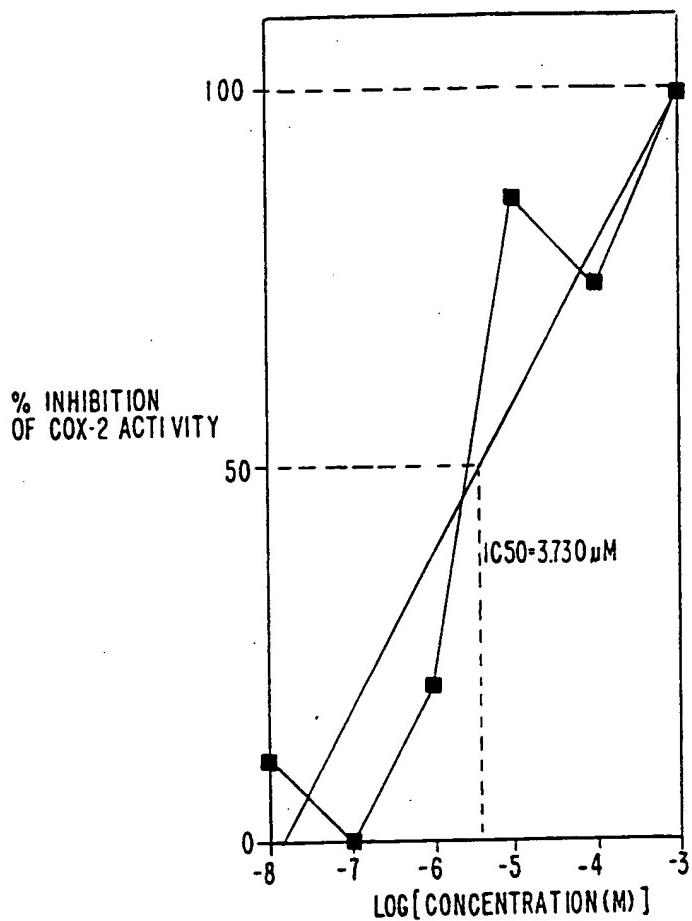
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FIG. 36G



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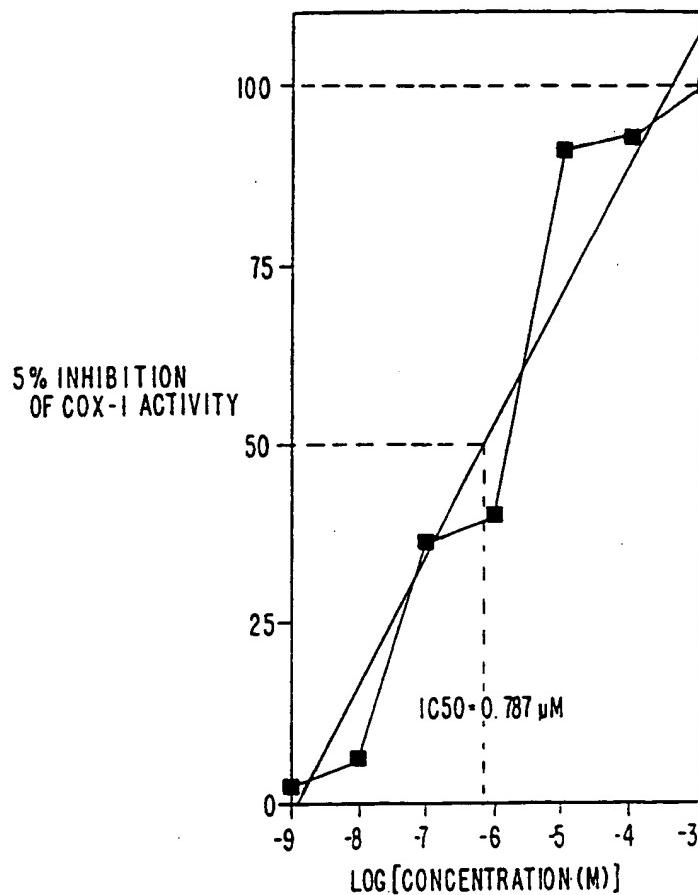
FIG. 36H



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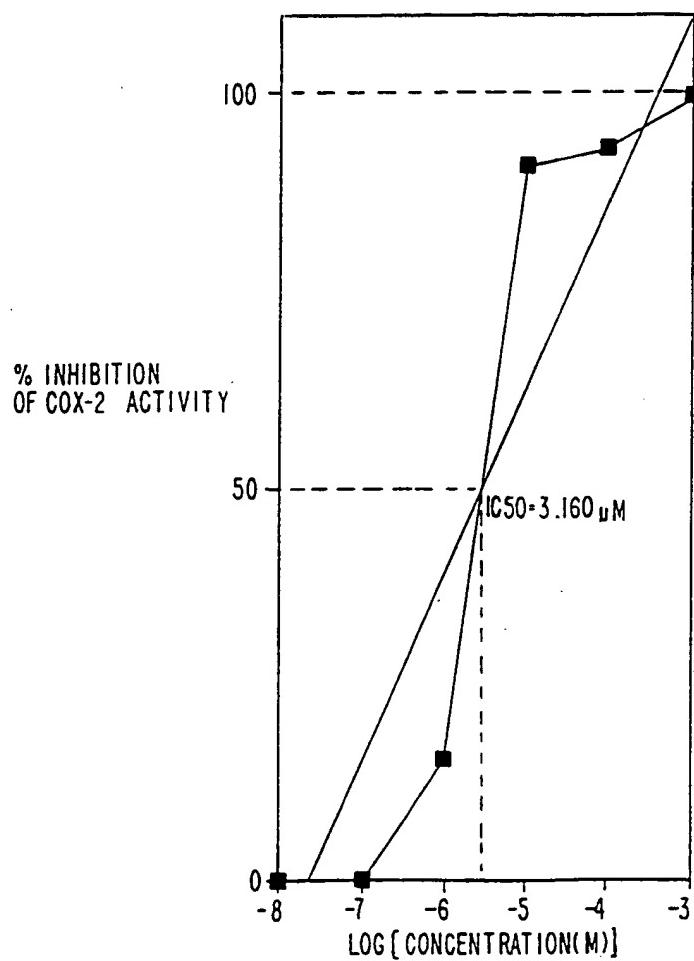
FIG. 36I



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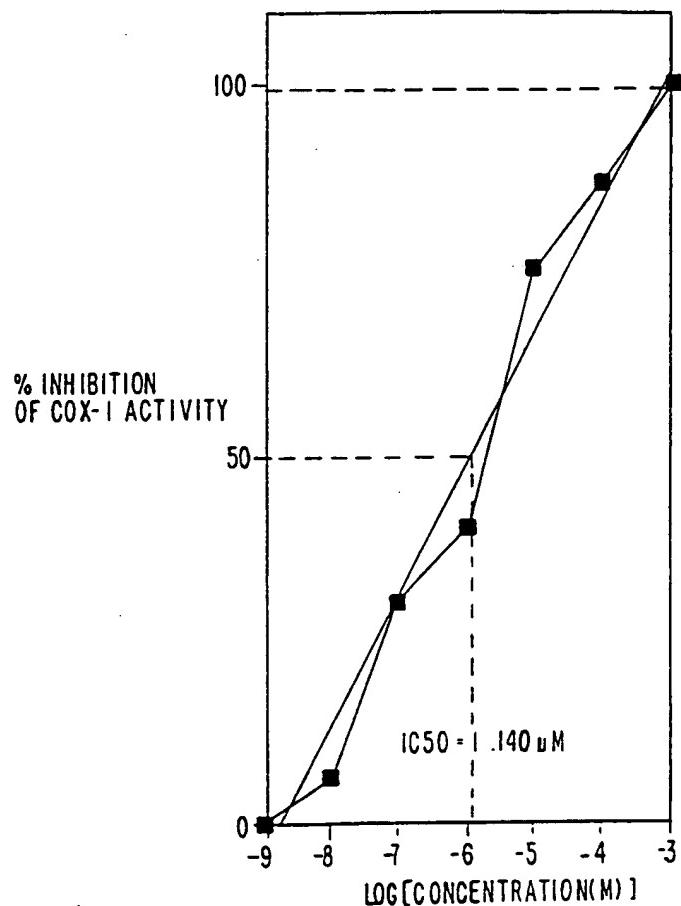
FIG. 36J



SUBSTITUTE SHEET (RULE 26)

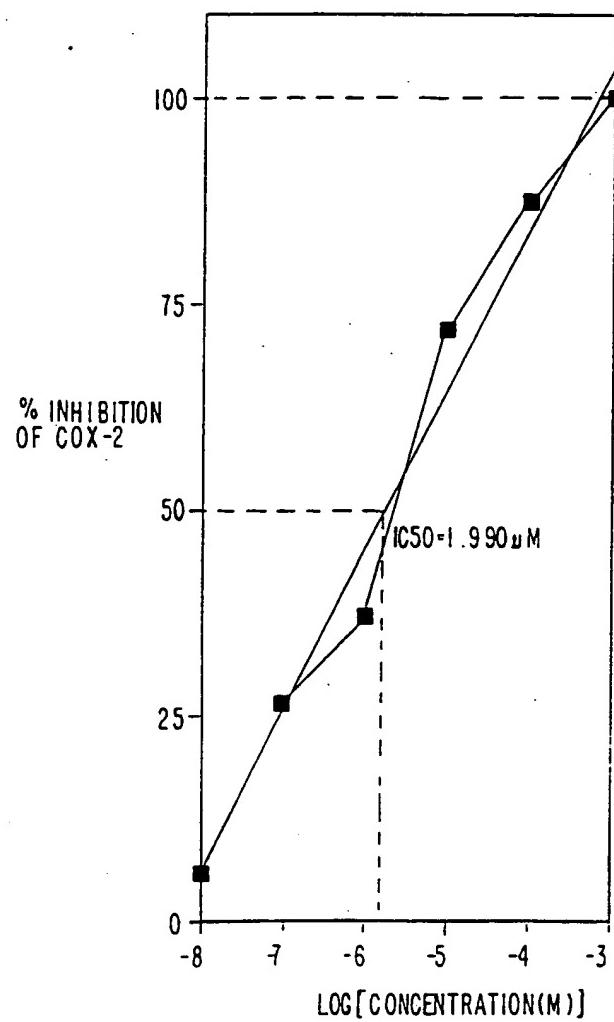
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FIG. 36K



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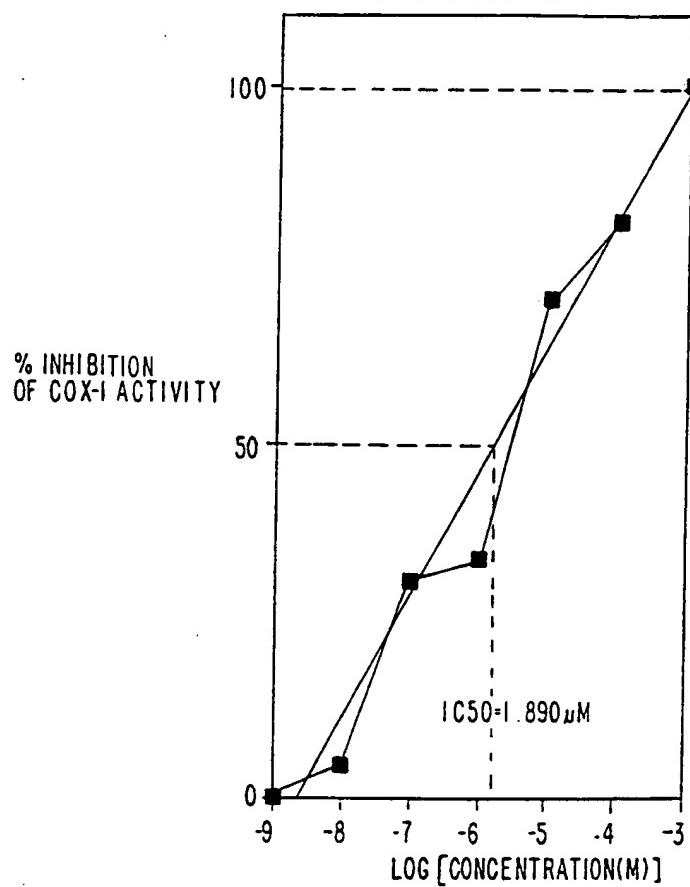
FIG. 36L



SUBSTITUTE SHEET (RULE 26)

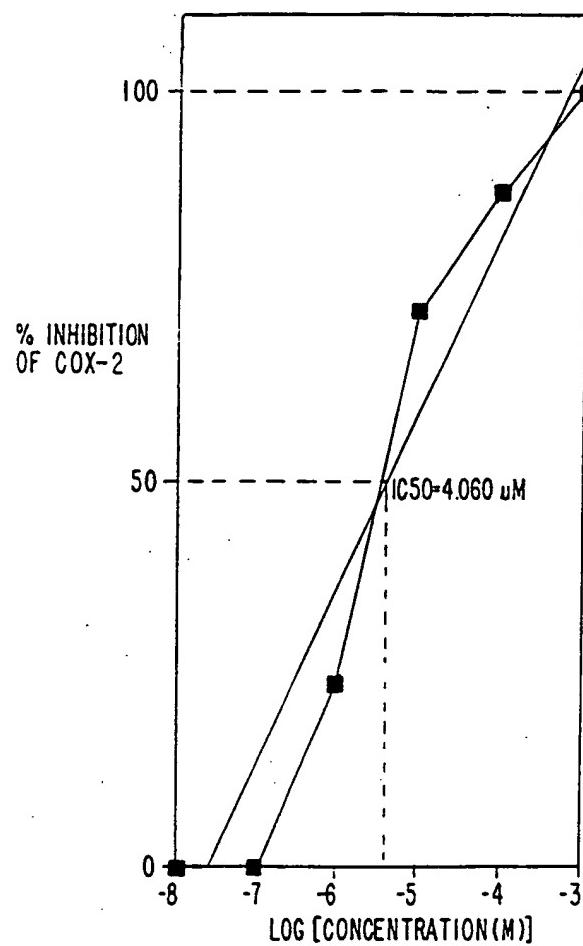
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FIG. 36M



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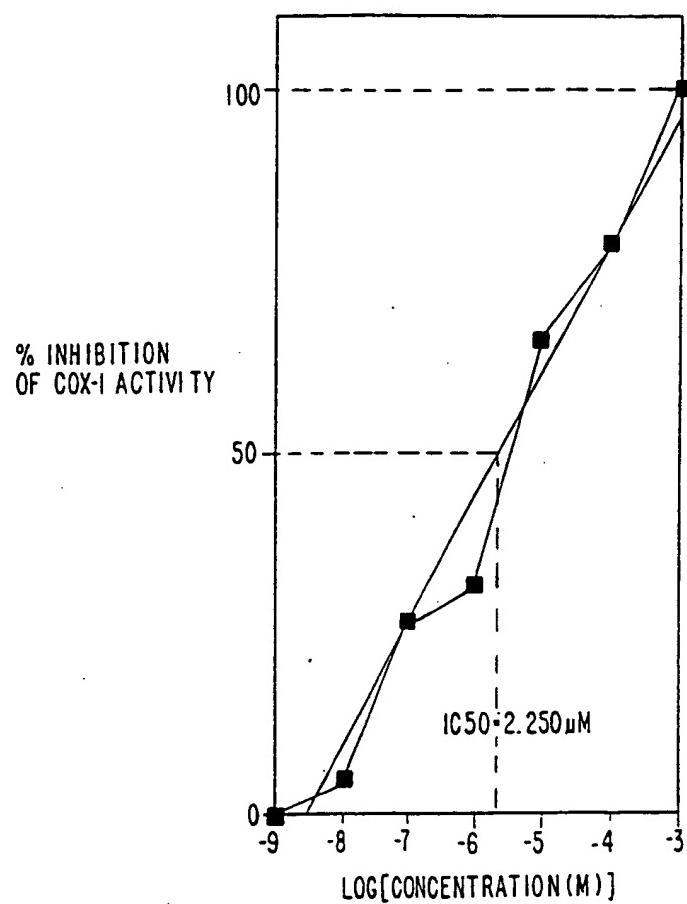
FIG. 36N



SUBSTITUTE SHEET (RULE 26)

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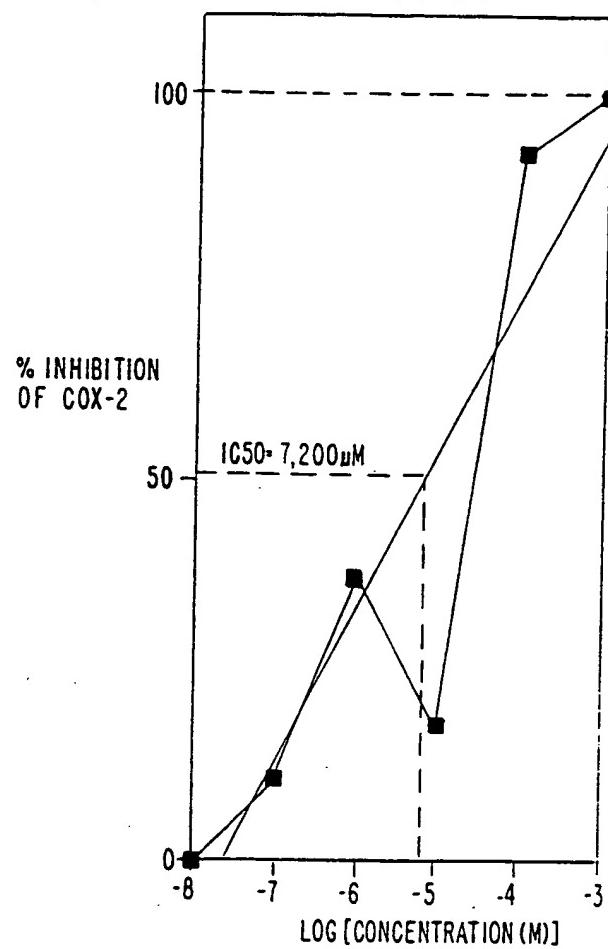
FIG. 360



SUBSTITUTE SHEET (RULE 26)

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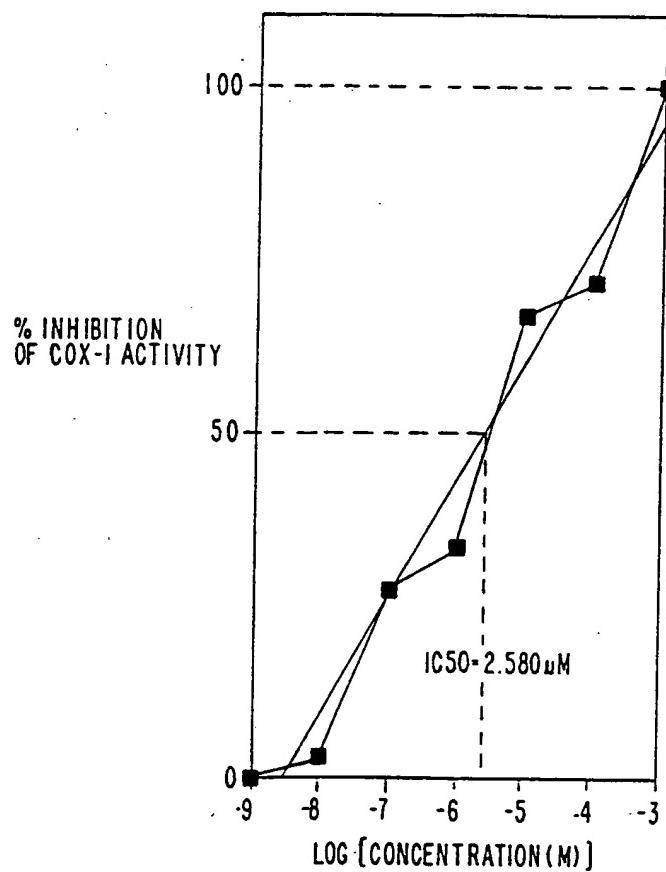
FIG. 36P



SUBSTITUTE SHEET (RULE 26)

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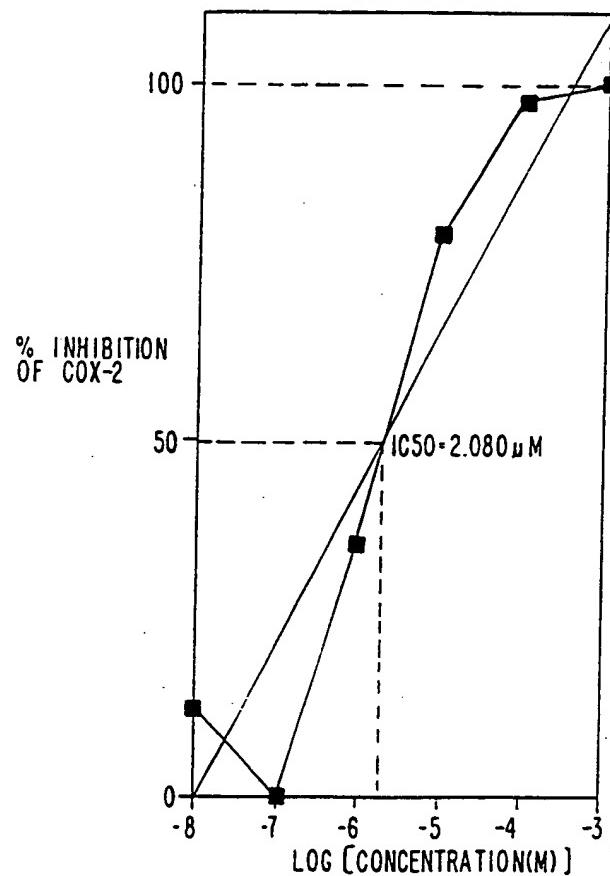
FIG. 36Q



SUBSTITUTE SHEET (RULE 26)

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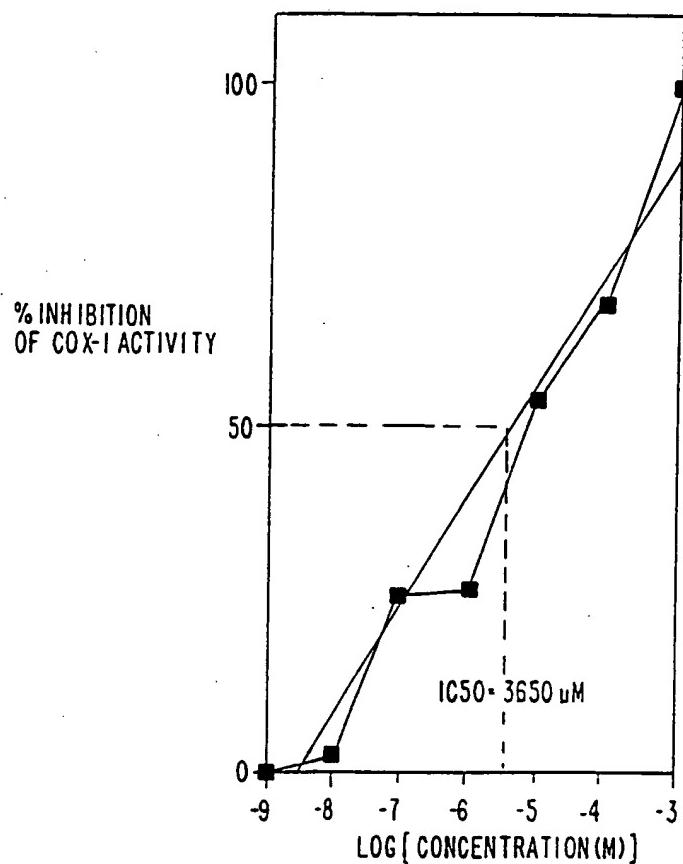
FIG. 36R



SUBSTITUTE SHEET (RULE 26)

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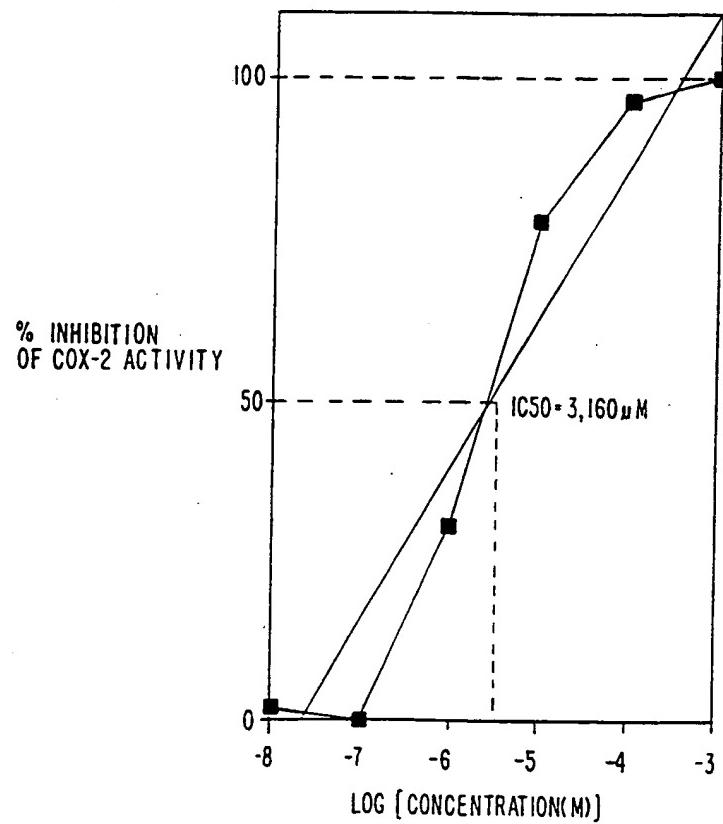
FIG. 36S



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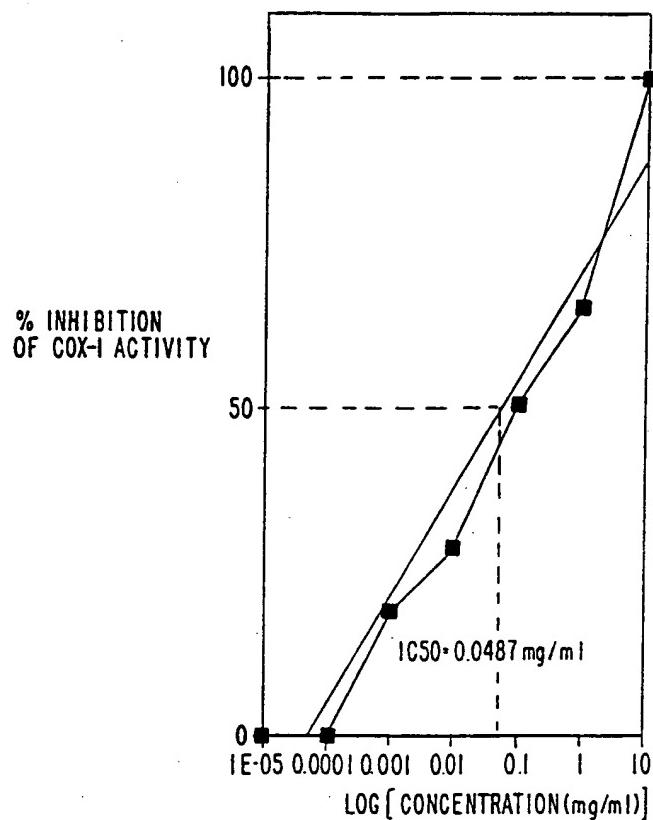
FIG. 36T



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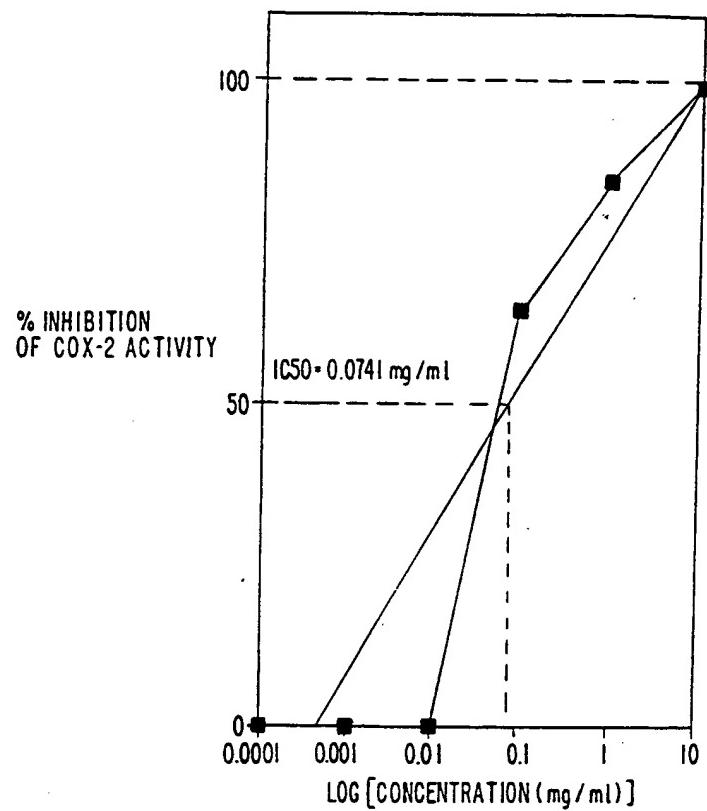
FIG. 36U



SUBSTITUTE SHEET (RULE 26)

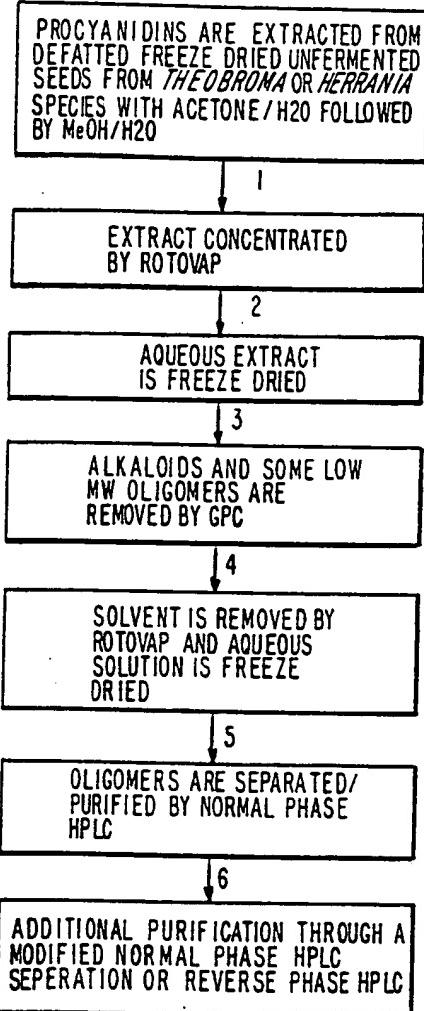
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FIG. 36V



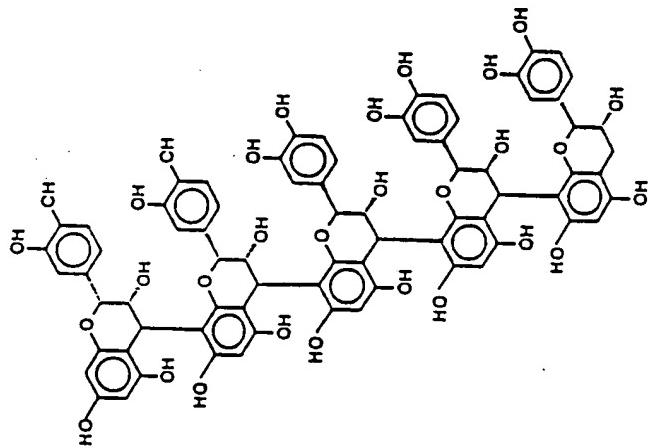
SUBSTITUTE SHEET (RULE 26)

FIG. 37

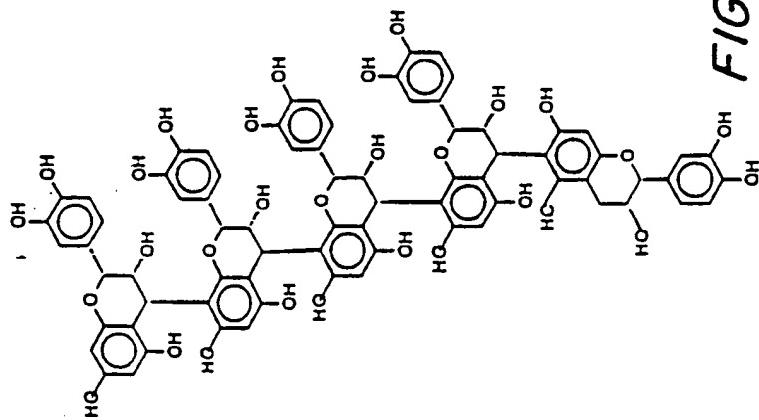


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F/G. 38B
 $(4\text{-}8)(4\text{-}8)(4\text{-}8)(4\text{-}6)$ CENNTAMER

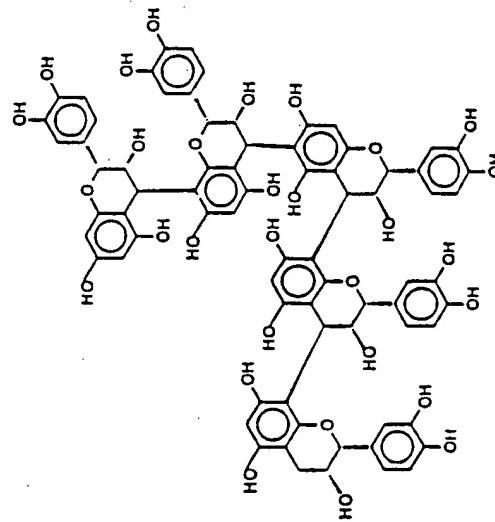


F/G. 38A
 $(4\text{-}8)(4\text{-}8)(4\text{-}8)(4\text{-}6)$ PENTAMER



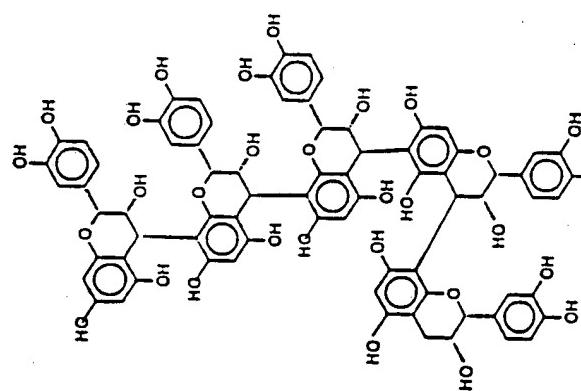
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(4-8)(4-6)(4-8)(4-6)PENTAMER

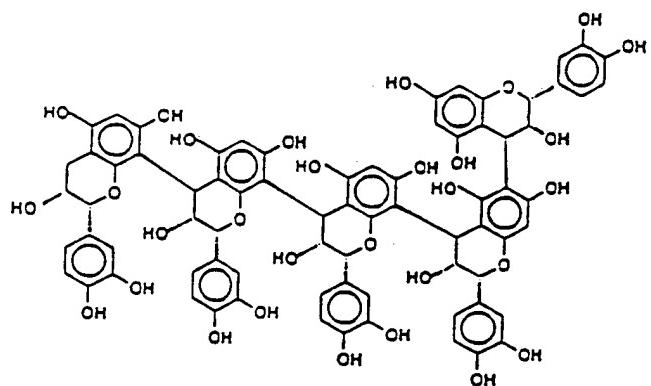
FIG. 38D



(4-8)(4-8)(4-6)(4-6)6PENTAMER

FIG 38C

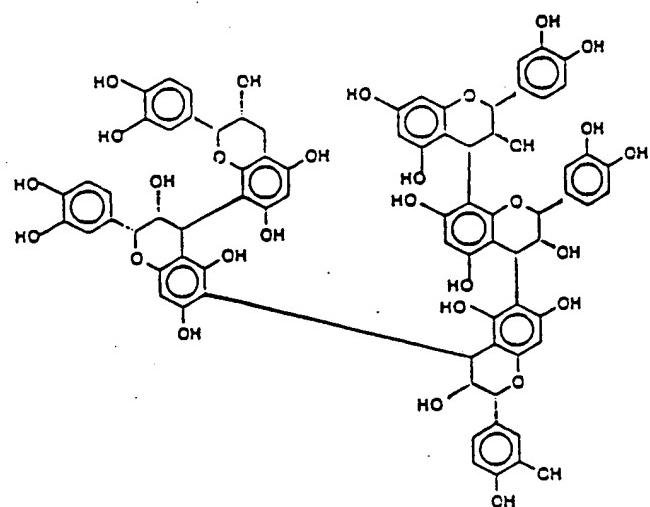
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(4-6)(4-8)(4-8)(4-8) PENTAMER

FIG. 38E

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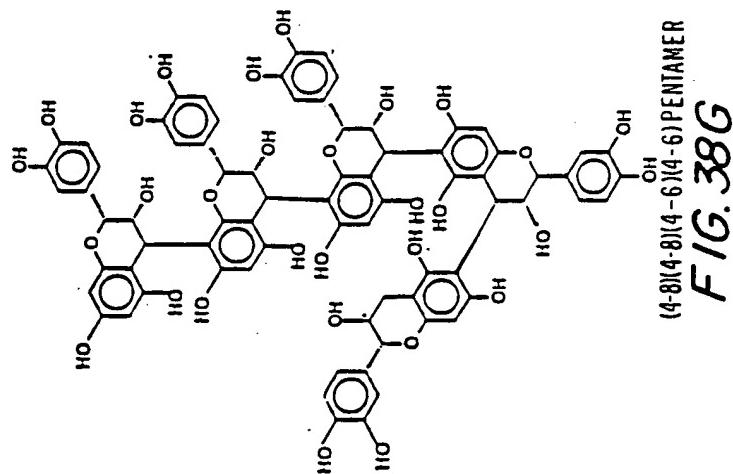
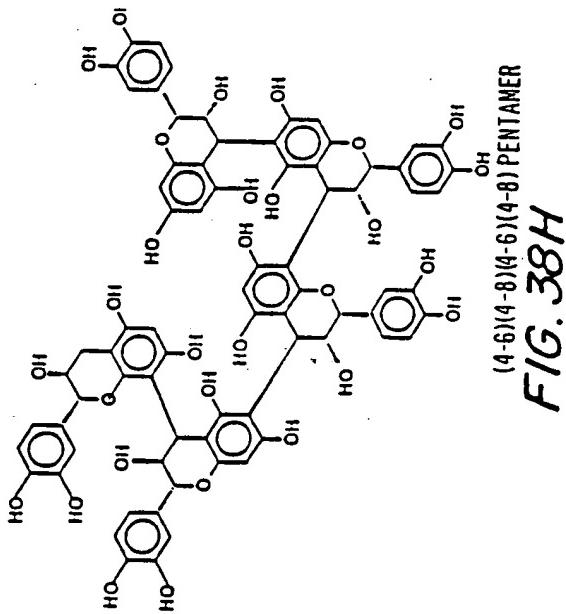


(4-8)(4-6)(4-6)(4-8) PENTAMER

FIG. 38F

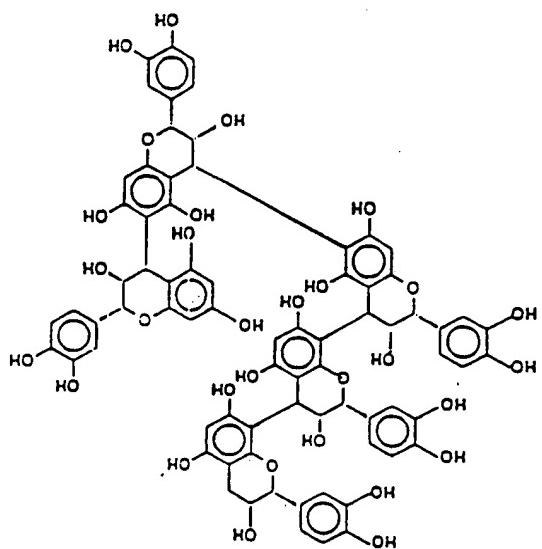
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(4-6)(4-6)(4-8)(4-8)PENTAMER

FIG. 38I

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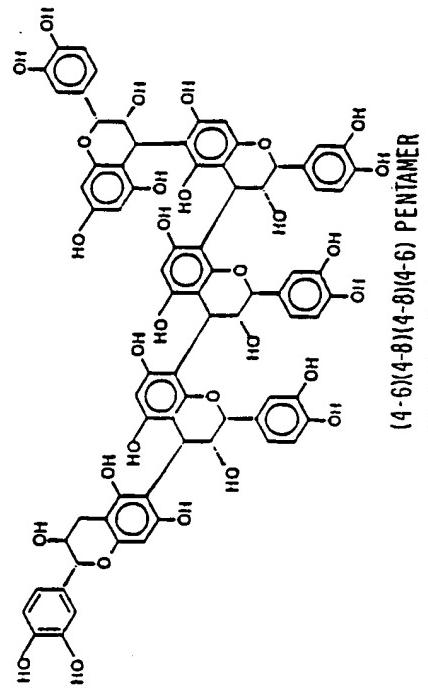


FIG. 38K

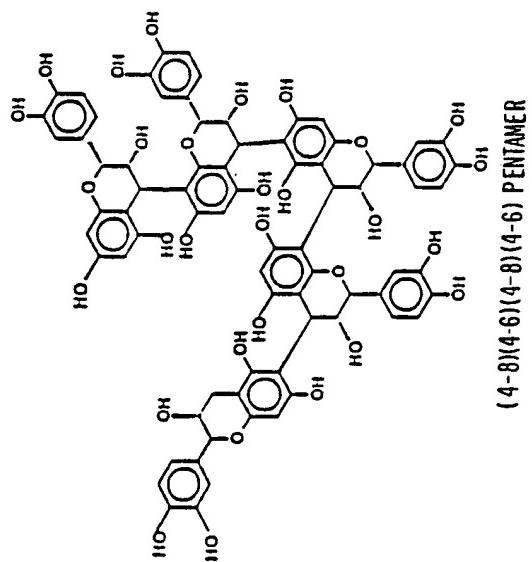
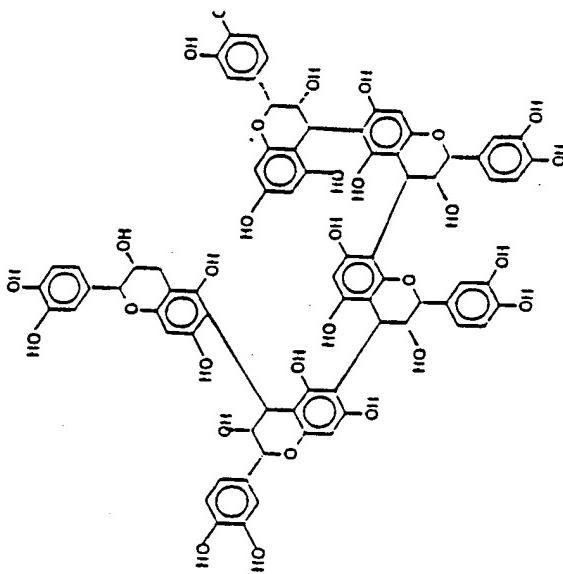


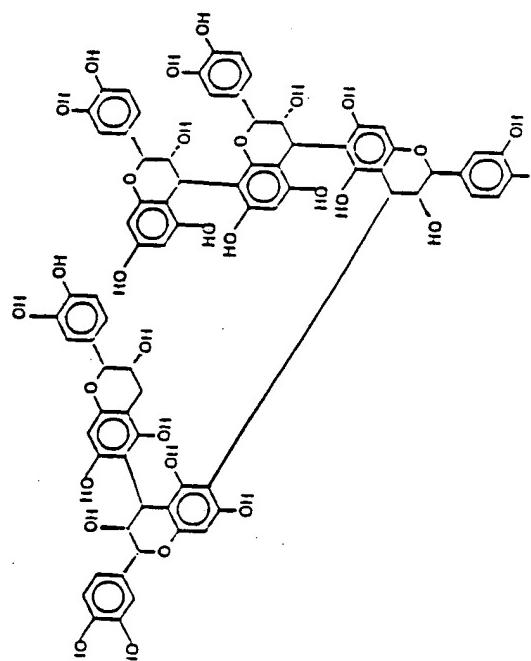
FIG. 38J

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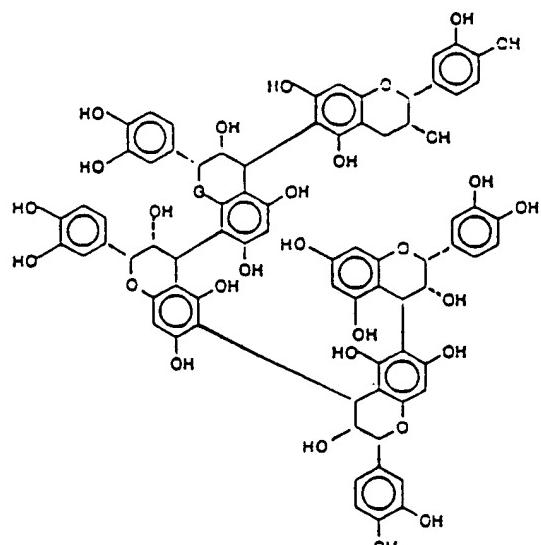
F/G.38M



F/G.38L

SUBSTITUTE SHEET (RULE 26)

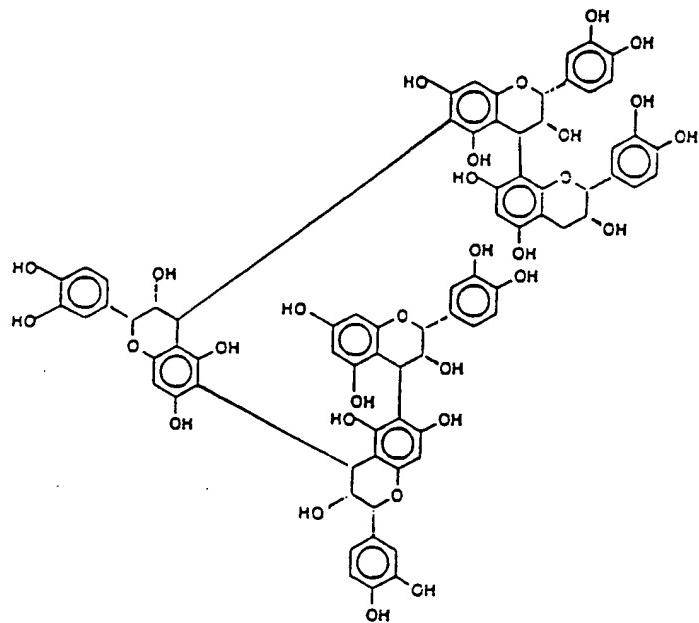
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(4-6)(4-6)(4-8)(4-6)PENTAMER

FIG. 38N

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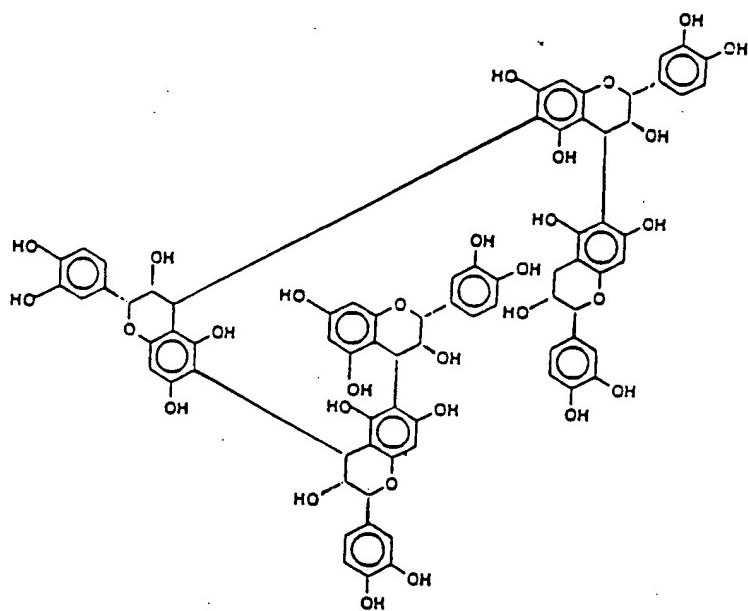


(4-6)(4-6)(4-6)(4-6)PENTAMER

FIG. 380

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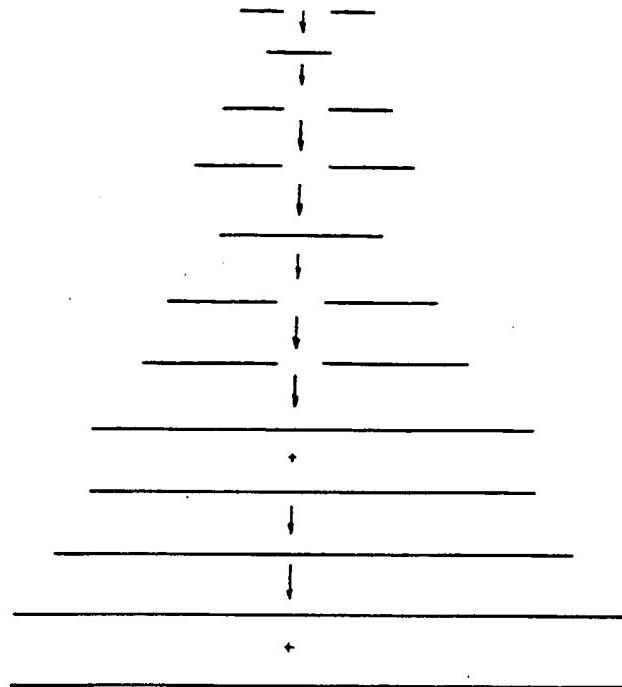
(4-6)(4-6)(4-6)(4-8)PENTAMER

FIG. 38P

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FIG. 39A



SUBSTITUTE SHEET (RULE 26)

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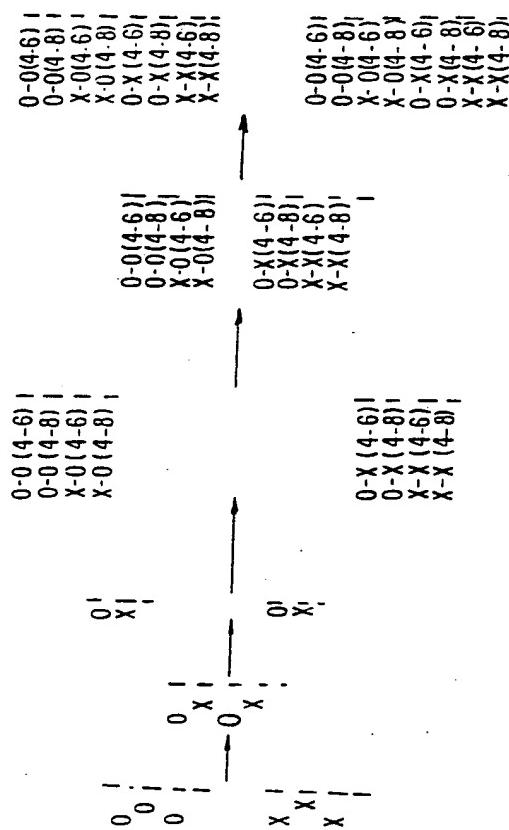
X-CAT
0-EC

(CONTINUE LEVEL V)

FIG. 39AA

SUBSTITUTE SHEET (RULE 26)

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X-CAT
0-ECF/G 39B
LEVEL I

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X-CAT
O-EC

(LEVEL 1)

FIG. 39C

SUBSTITUTE SHEET (RULE 26)

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X-CAT
0-EC

FIG. 39D

X-X-X {4-8; 4-6};
X-X-X {4-8; 4-6};

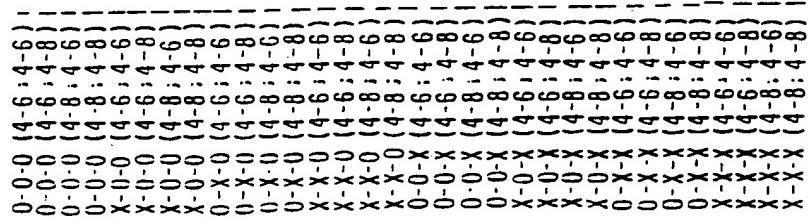
SUBSTITUTE SHEET (RULE 26)

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X-CAT
O-EC

(LEVEL II)

FIG. 39E



SUBSTITUTE SHEET (RULE 26)

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X-CAT
0-EC

(CONTINUE- LEVEL II)

F/G. 39F

0-0-0 (4-6;4-6)
0-0-0 (4-6;4-8)
0-0-0 (4-8;4-6)
0-0-0 (4-8;4-8)
0-0-0 (4-6;4-6)
0-0-0 (4-6;4-8)
0-0-0 (4-8;4-6)
0-0-0 (4-8;4-8)
X-0-0 (4-6;4-6)
X-0-0 (4-6;4-8)
X-0-0 (4-8;4-6)
X-0-0 (4-8;4-8)
X-0-0 (4-6;4-6)
X-0-0 (4-6;4-8)
X-0-0 (4-8;4-6)
X-0-0 (4-8;4-8)
0-X-0 (4-6;4-6)
0-X-0 (4-6;4-8)
0-X-0 (4-8;4-6)
0-X-0 (4-8;4-8)

SUBSTITUTE SHEET (RULE 26)

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X = CAT
0 = EC

(LEVEL III)

FIG. 39G

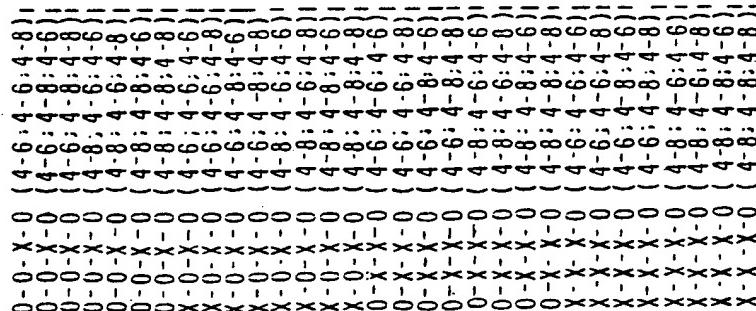
SUBSTITUTE SHEET (RULE 26)

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X-CAT
0-EC

(CONTINUE LEVEL III)

FIG. 39H



SUBSTITUTE SHEET (RULE 26)

161 | 222

X-CAT
0-EC

(CONTINUE ON LEVEL III)

$$X - X_0 - X_1(4-8; 4-8; 4-8);$$

FIG. 391

SUBSTITUTE SHEET (RULE 26)

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X-CAT
O-EC

(CONTINUE LEVEL III)

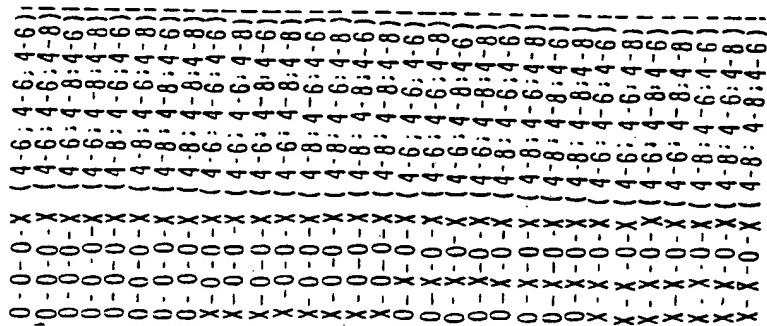


FIG. 39J

SUBSTITUTE SHEET (RULE 26)

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X-CAT
O-EC

FIG. 39K

$\times \times \times \times \times \times \times \times \times \times$

SUBSTITUTE SHEET (RULE 26)

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X = CAT
O = EC

(LEVEL IV)

FIG. 39L

SUBSTITUTE SHEET (RULE 26)

165 | 222

X-CAT
O-EC

(CONTINUE - LEVEL IV)

FIG. 39M

SUBSTITUTE SHEET (RULE 26)

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X=CAT
O=EC

(CONTINUE LEVEL IV)

FIG. 39N

SUBSTITUTE SHEET (RULE 26)

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X-CAT
O-EC

(CONTINUE LEVEL IV)

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X-CAT
0-EC

(CONTINUE LEVEL IV)

FIG. 39P

SUBSTITUTE SHEET (RULE 26)

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X = CAT
0 = EC

(CONTINUE LEVEL IV)

FIG. 39Q

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X = CAT
0 = EC

(CONTINUE LEVEL IV)

FIG. 39R

SUBSTITUTE SHEET (RULE 26)

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X-CAT
O-EC

(CONTINUE LEVEL IV)

FIG. 395

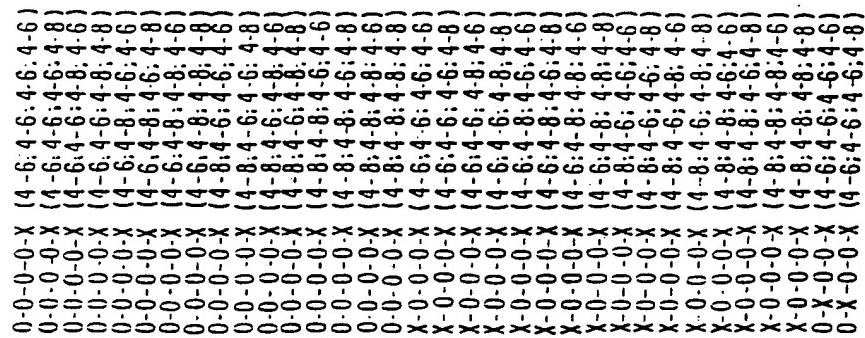
SUBSTITUTE SHEET (RULE 26)

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X-CAT
O-EC

(LEVEL V)

FIG. 39T



SUBSTITUTE SHEET (RULE 26)

173 | 222

X=CAT
O=EC

(CONTINUE LEVEL V)

FIG. 39U

SUBSTITUTE SHEET (RULE 26)

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X-CAT
O-EC

(CONTINUE LEVEL V)

FIG. 39V

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(CONTINUE LEVEL V)

FIG. 39W

SUBSTITUTE SHEET (RULE 26)

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X-CAT
0-SEC

(CONTINUE LEVEL V)

FIG. 39 X

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X = CAT
0 = EC

(CONTINUE LEVEL V)

FIG. 39Y

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X-CAT
0-EC

(CONTINUE LEVEL V)

FIG. 39Z

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FIG. 40A

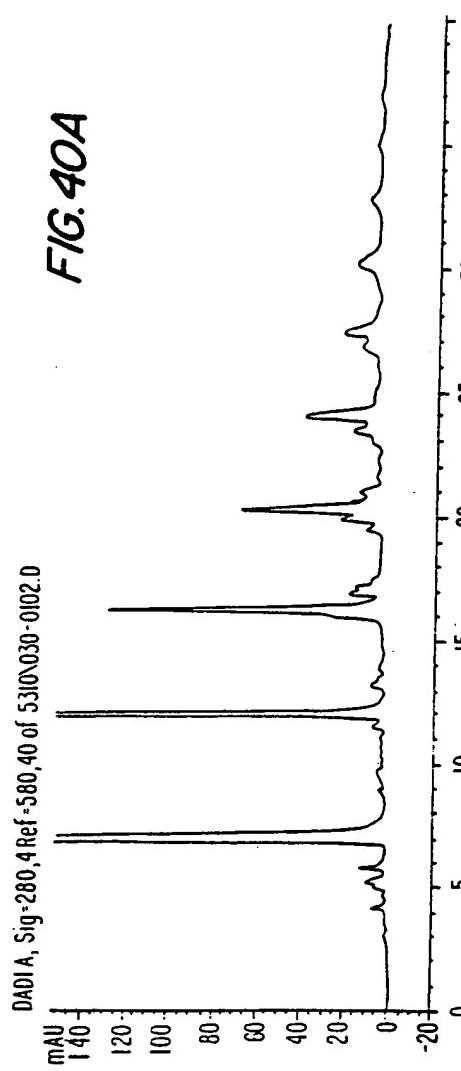
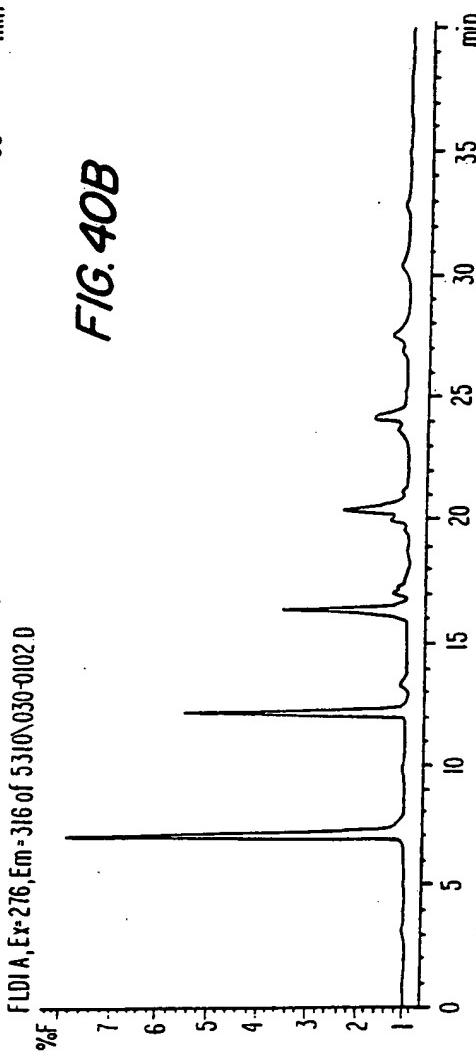


FIG. 40B



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FIG. 4/A

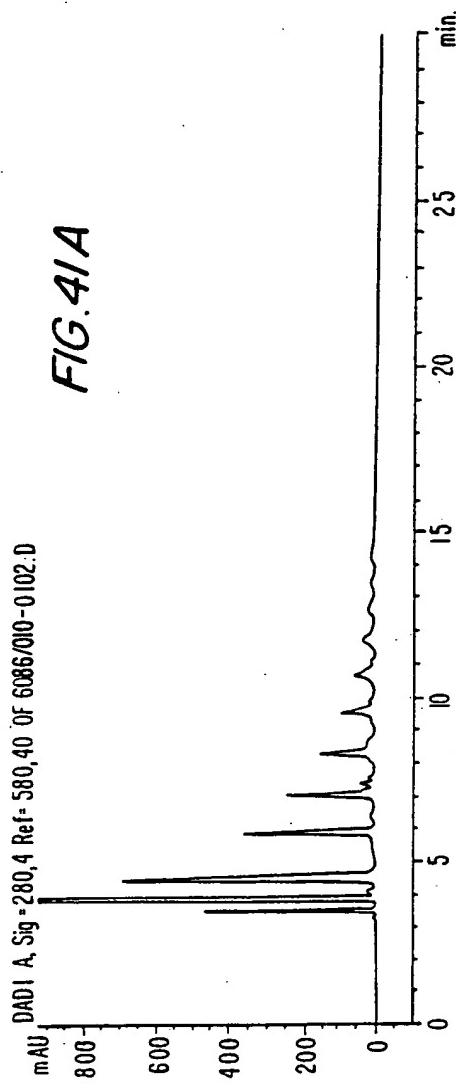
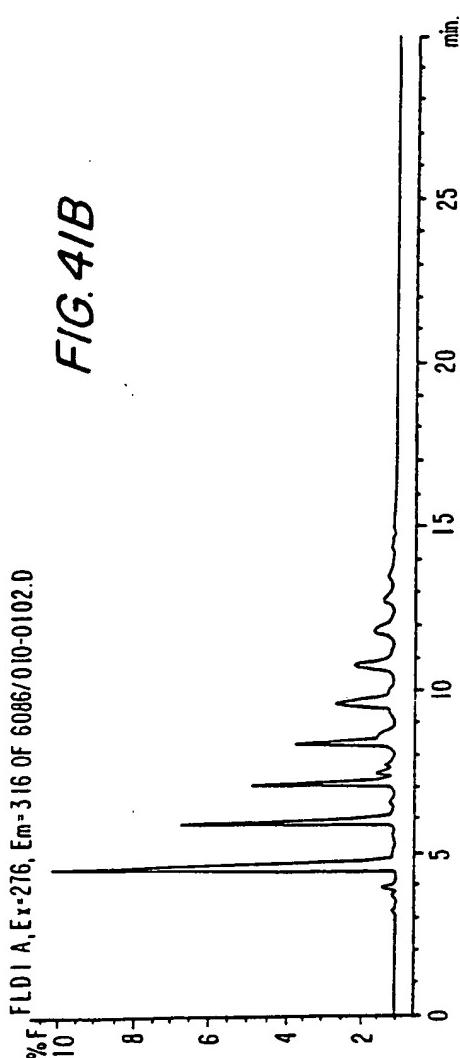
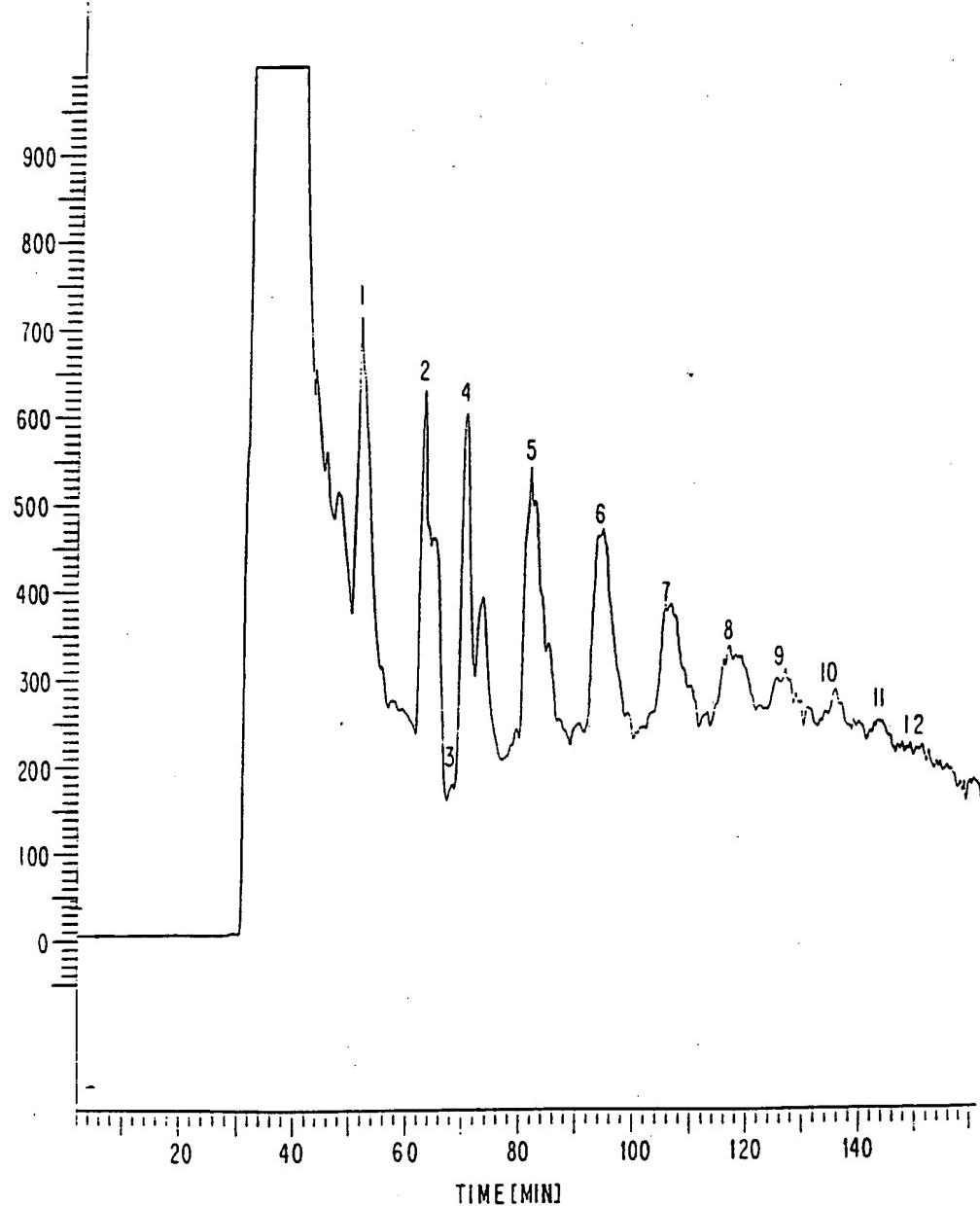


FIG. 4/B



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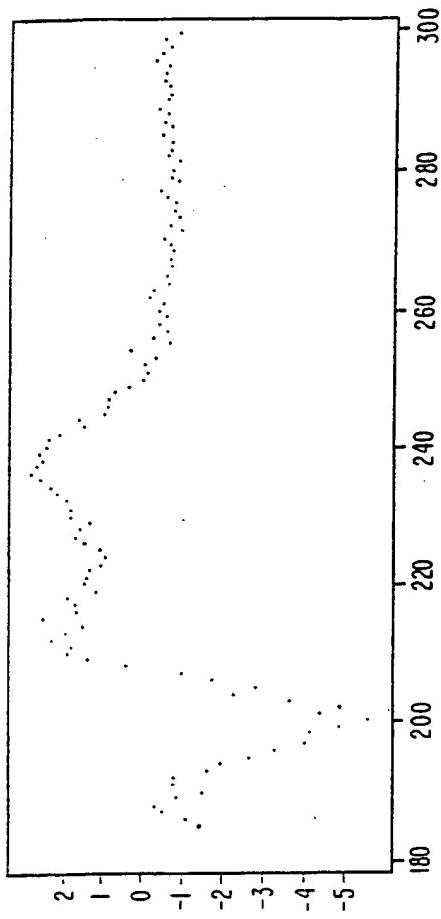
FIG.42



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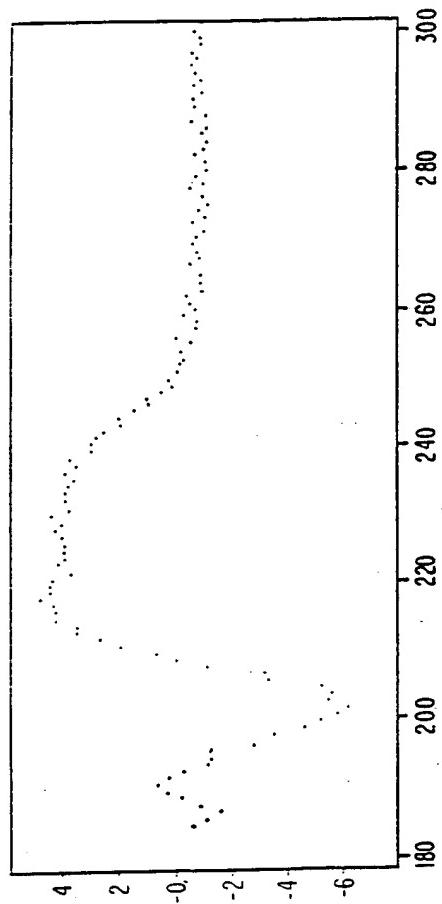
FIG. 43A



SUBSTITUTE SHEET (RULE 26)

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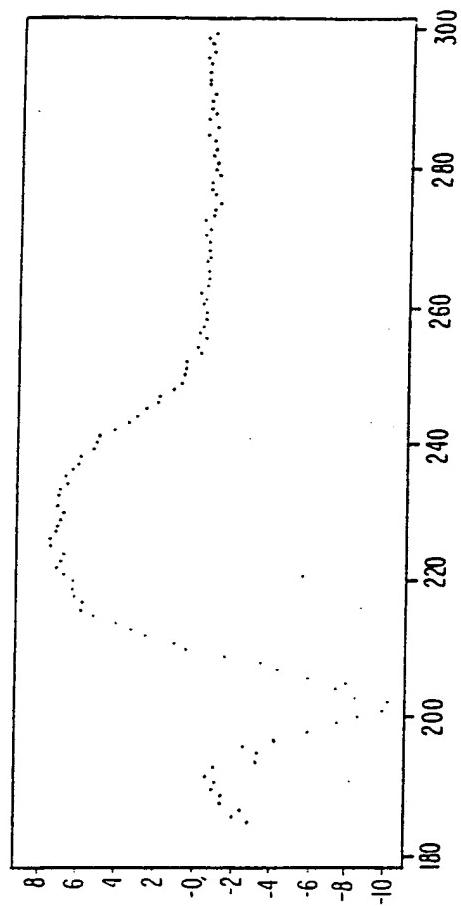
FIG. 43B



SUBSTITUTE SHEET (RULE 26)

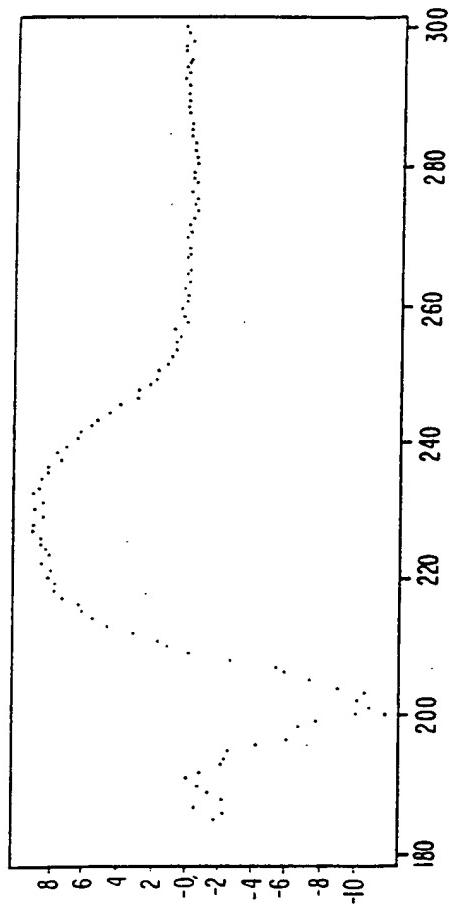
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FIG. 43C



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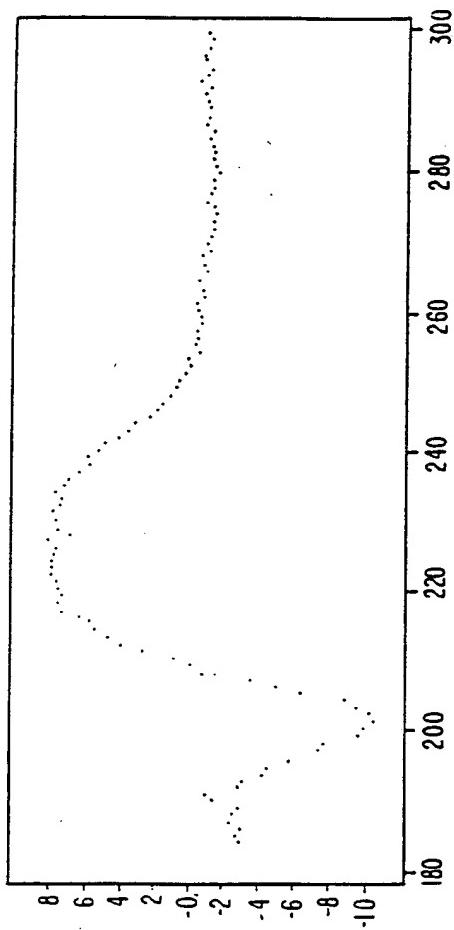
FIG. 43D



SUBSTITUTE SHEET (RULE 26)

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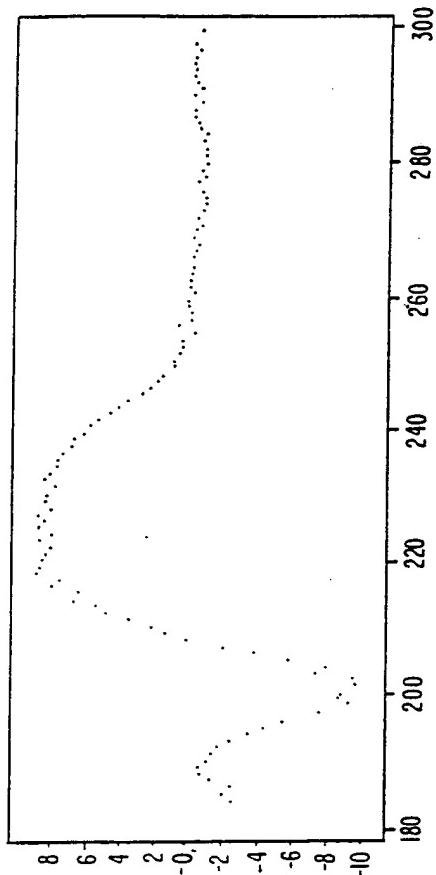
FIG. 43E



SUBSTITUTE SHEET (RULE 26)

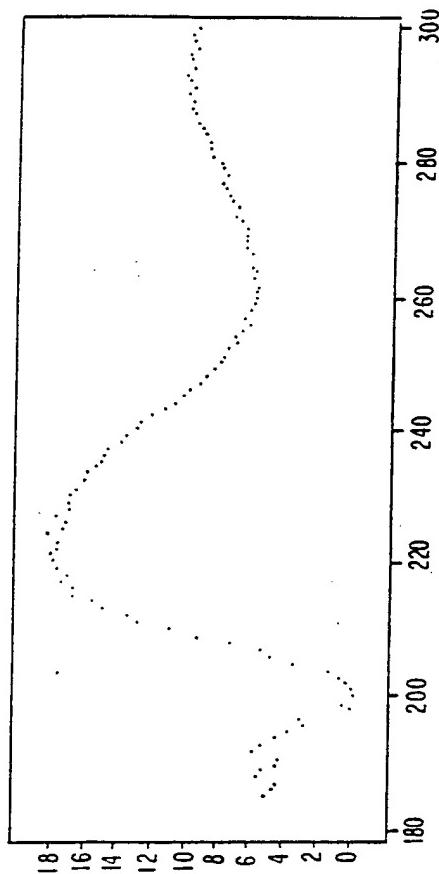
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FIG. 43F

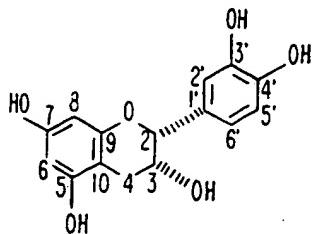


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FIG. 43G



SUBSTITUTE SHEET (RULE 26)

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EPICATECHIN			
¹ H	CHEMICAL SHIFT (ppm)	¹³ C	CHEMICAL SHIFT (ppm)
2	4.81	2	79.84
3	4.16	3	67.46
4α 4β	{ 2.73 2.85	4 6	29.24 95.87
6	5.94	8	96.36
8	5.91	2'	115.29
5' 6'	{ 6.75 6.79	5' 6'	{ 115.88 119.39
2'	6.97		

FIG. 44A

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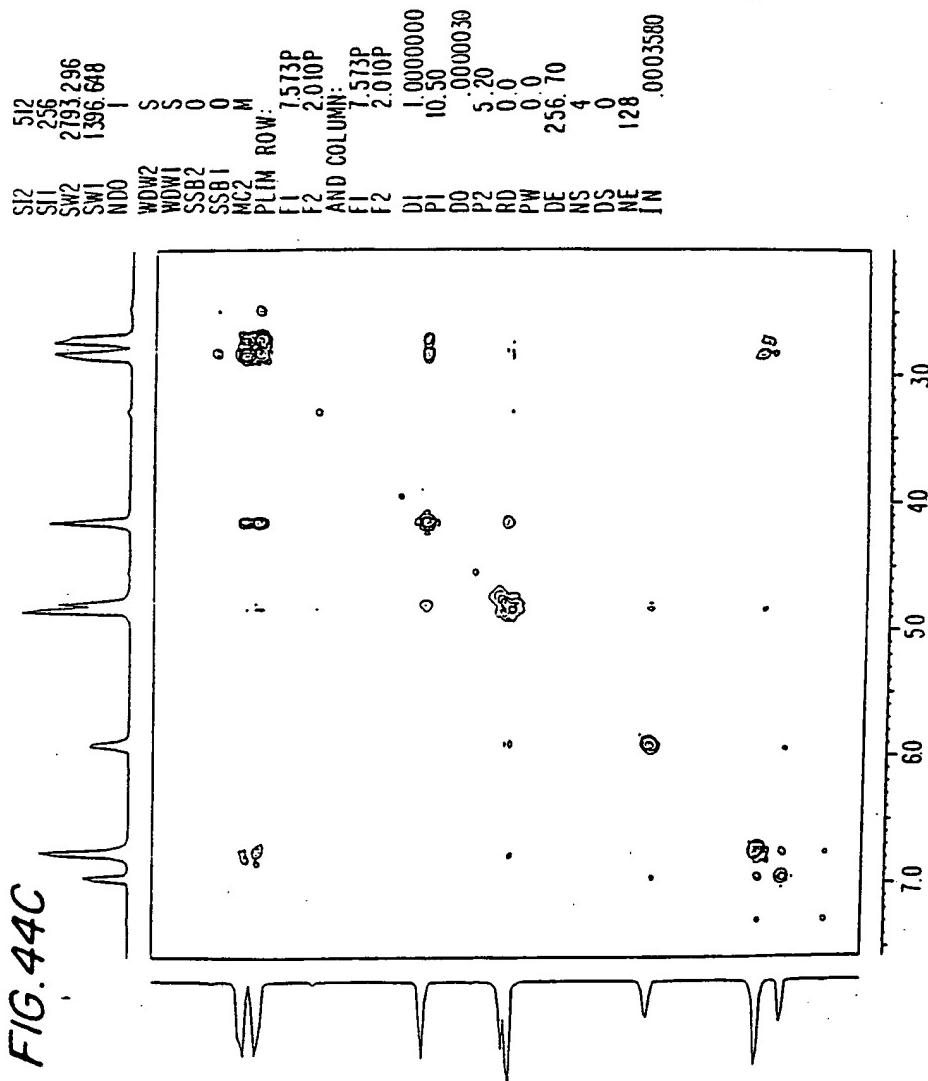
FIG. 44B

FIG. 44B

Peak Label	Chemical Shift (ppm)
S1	30H
SOLVENT	CDC13
SF	125.759
SF0	125.770
SF02	500.130
SF2	125.759
SY	93.0
S1	364.000
S1D	65.536
SW	294.11765
HZ/PT	.898
VD	.0080
PW	0.0
RD	0.0
AQ	1.14
RG	100
NS	128
TE	300
DE	23.8
DR	12
DW	17
FW	36800
IP	0.2
30H	9912.000
IP'	1.0
IP'	1.000
GB	0.0
NC	2
CX	20.00
CY	5.00
F1	170.005P
F2	20.009P
M1	0.0
DC	1.000
HZ/CH	943.73
HZ/N/CM	7.500
IS	-
SR	-10527.08
DI	1.0000000

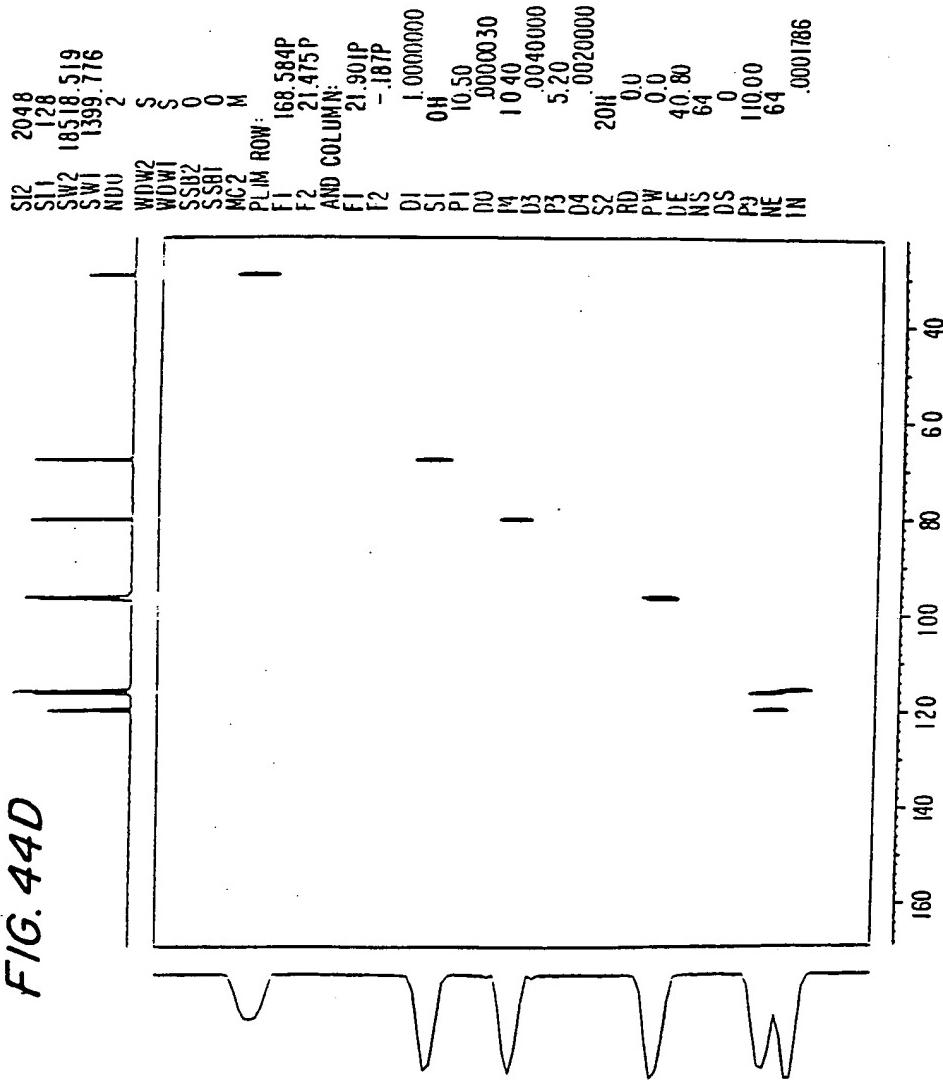
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SUBSTITUTE SHEET (RULE 26)

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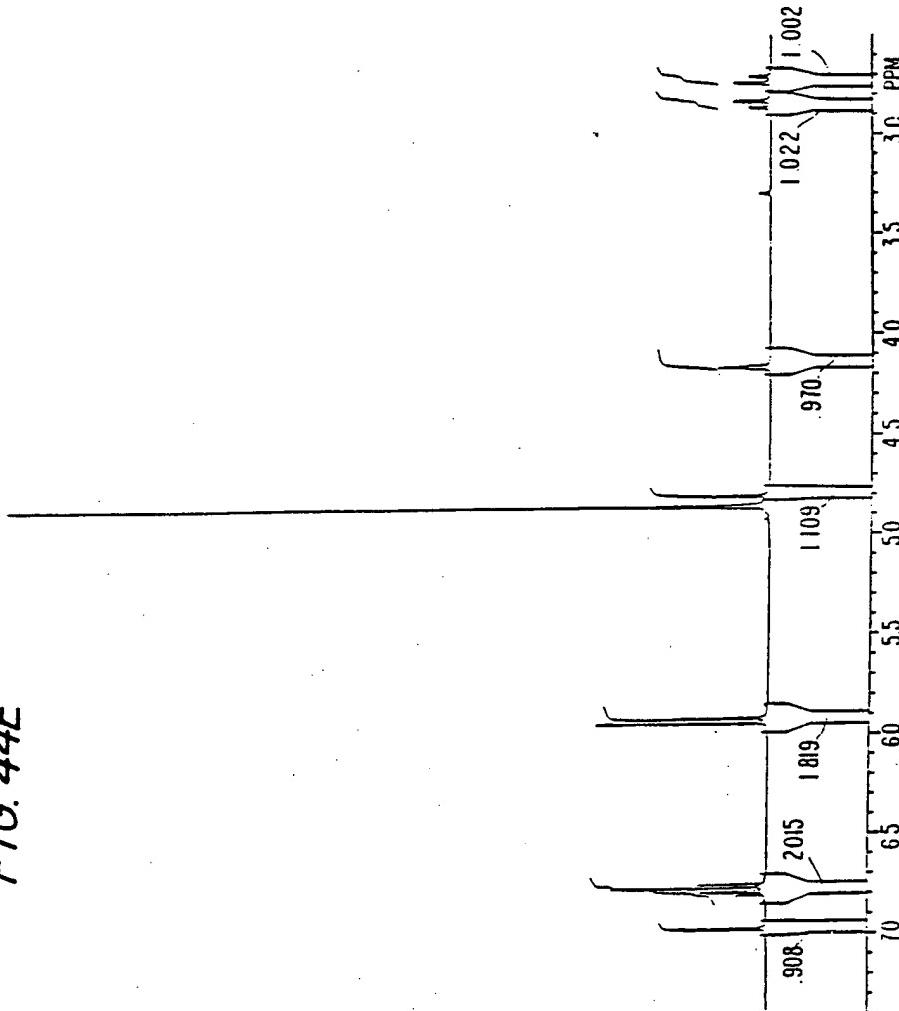


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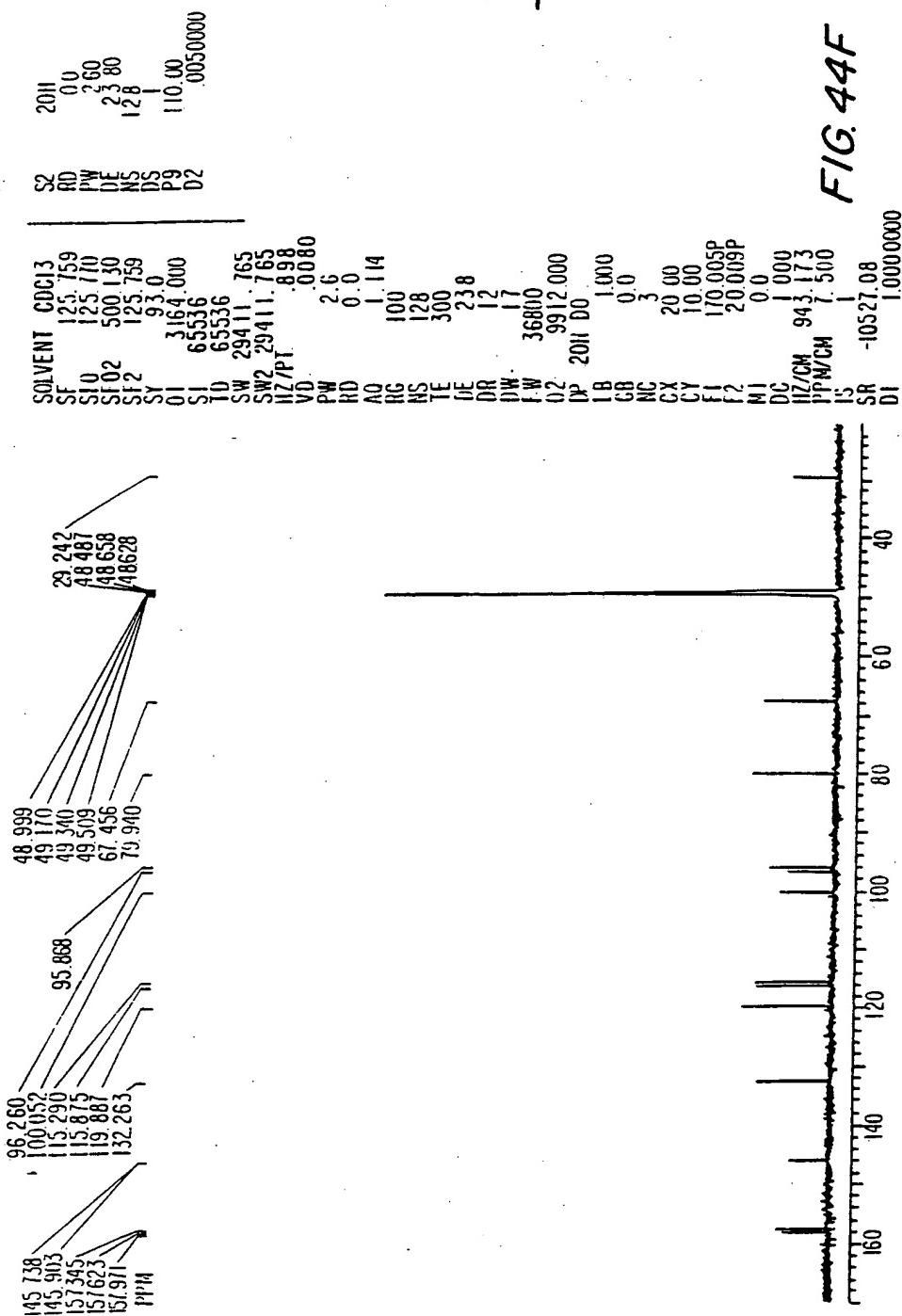
SOLVENT	0DC3
SF	500.137
SFO	500.136
S102	500.130
S12	500.137
SY	166.0
O1	3894.590
S1D	3216.8
SW	6024.096
H2/PT	6021.096
VD	368
PW	0.0
RD	0.0
AQ	2.120
RG	8
NS	16
TE	300
DE	106.3
DR	12
DW	0.3
FW	7600
DP	0.2
631P0	50000000
CD	0.0
NC	0
CX	2000
CY	1500
F1	7.500P
I2	2.500P
MJ	0.0
DC	1.000
H2/CM	125.030
PPM/CM	.250
IS	3
SR	7394.06

FIG. 44E



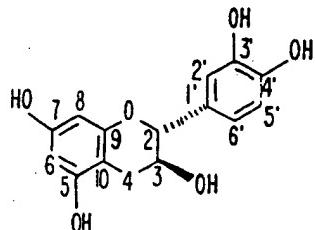
SUBSTITUTE SHEET (RULE 26)

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SUBSTITUTE SHEET (RULE 26)

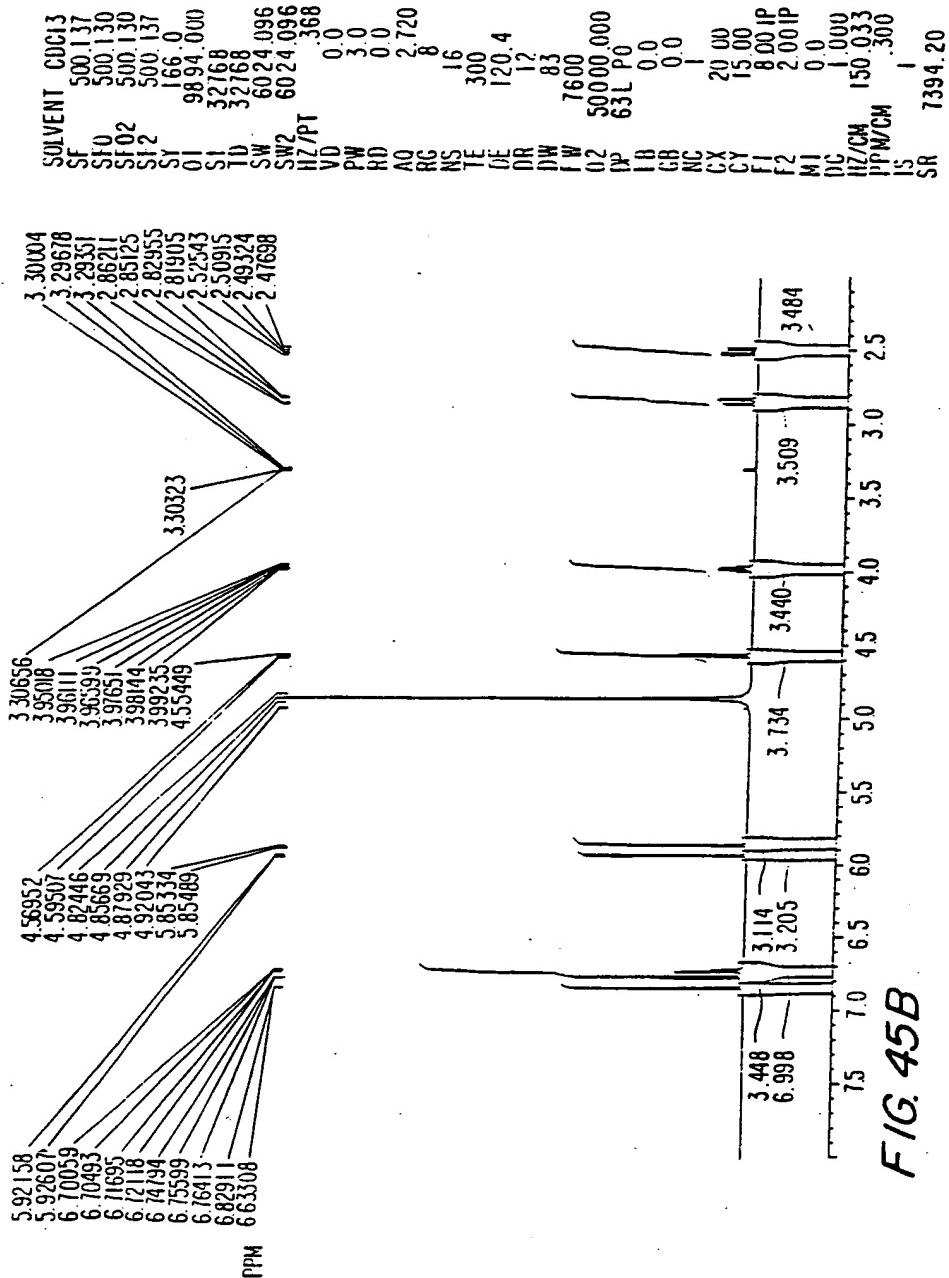
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¹ H	CHEMICAL SHIFT (ppm)	¹³ C	CHEMICAL SHIFT (ppm)
2	4.56	2	79.84
3	3.97	3	67.46
4 _α }	{ 2.50	4	29.24
4 _β }	{ 2.84	6	95.87
6	5.85	8	96.36
8	5.92	2'	115.29
2'	6.83	6'	{ 116.08
5'	{ 6.76	5'	{ 120.08
6'	{ 6.71		

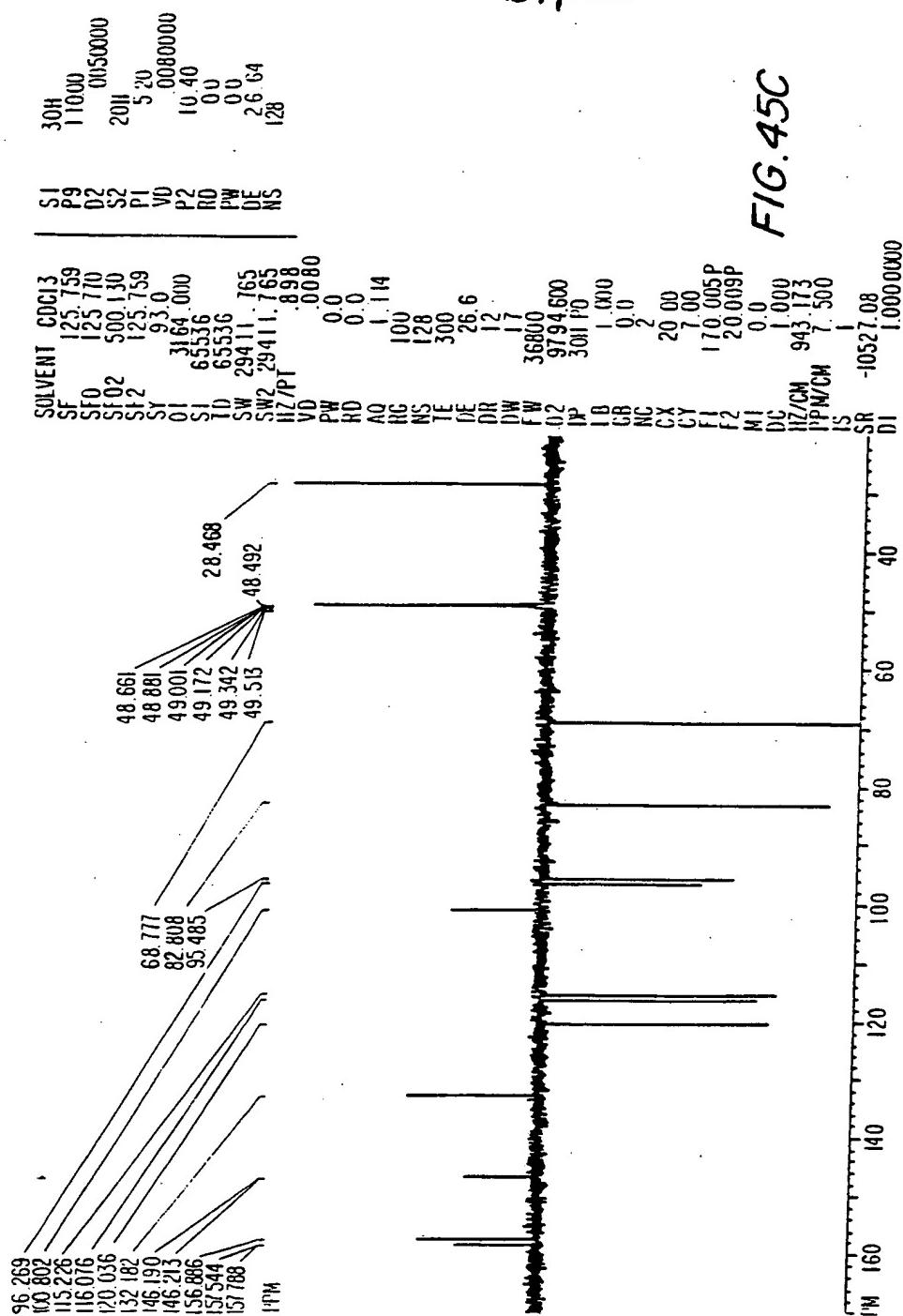
FIG. 45A

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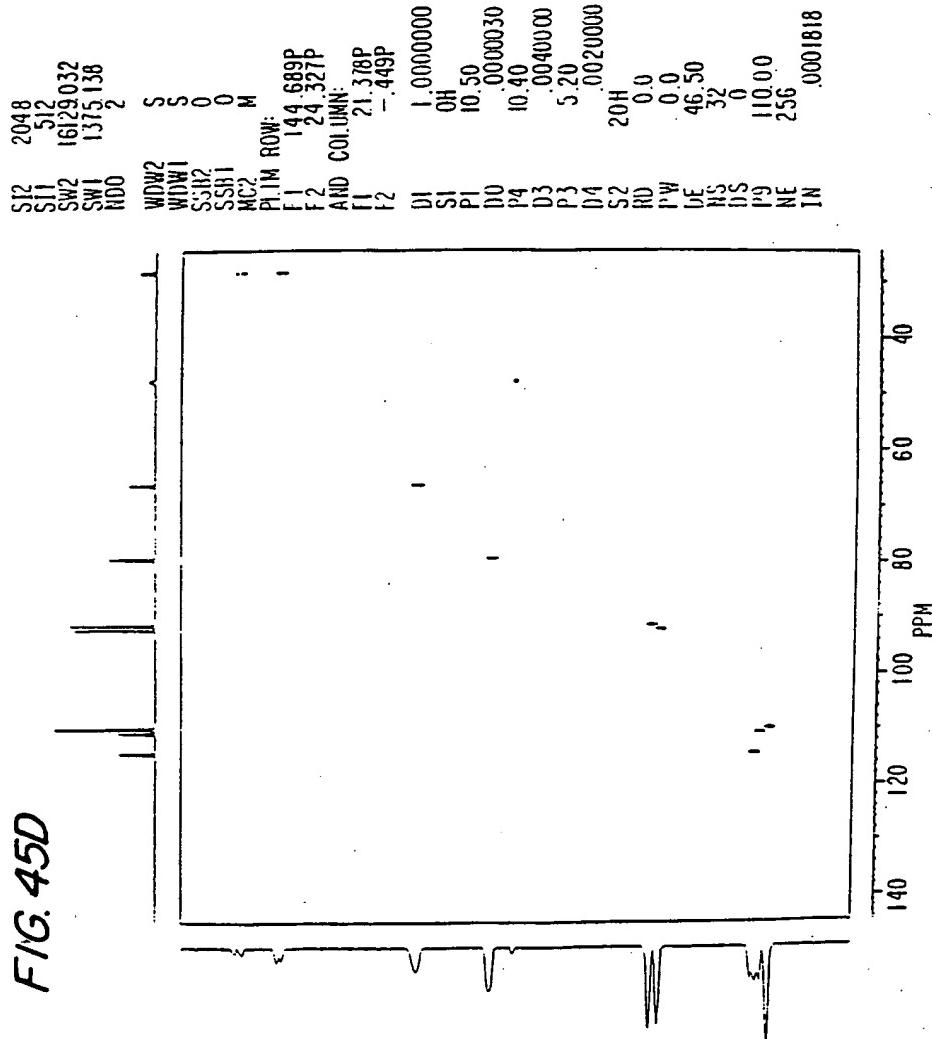


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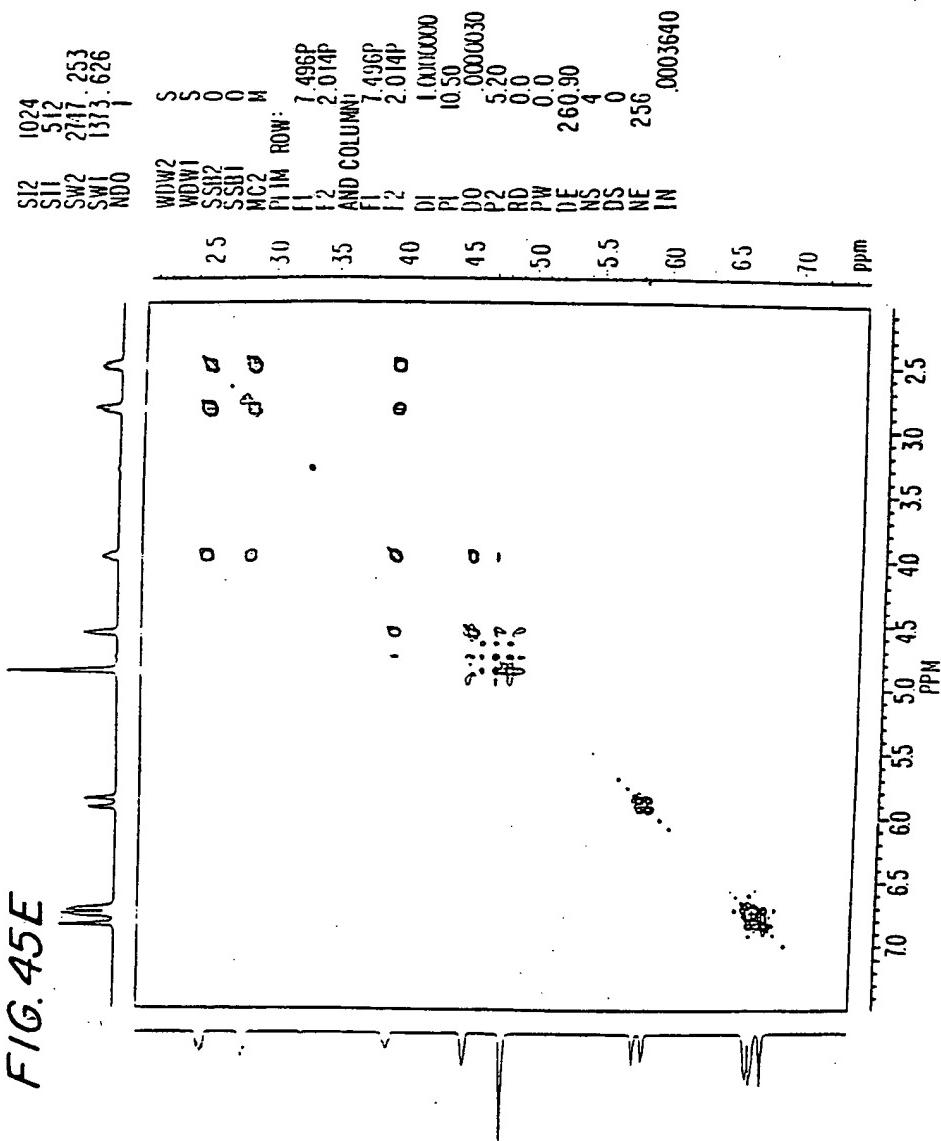


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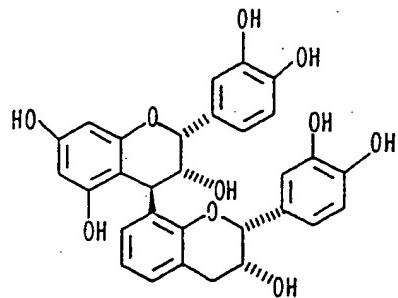


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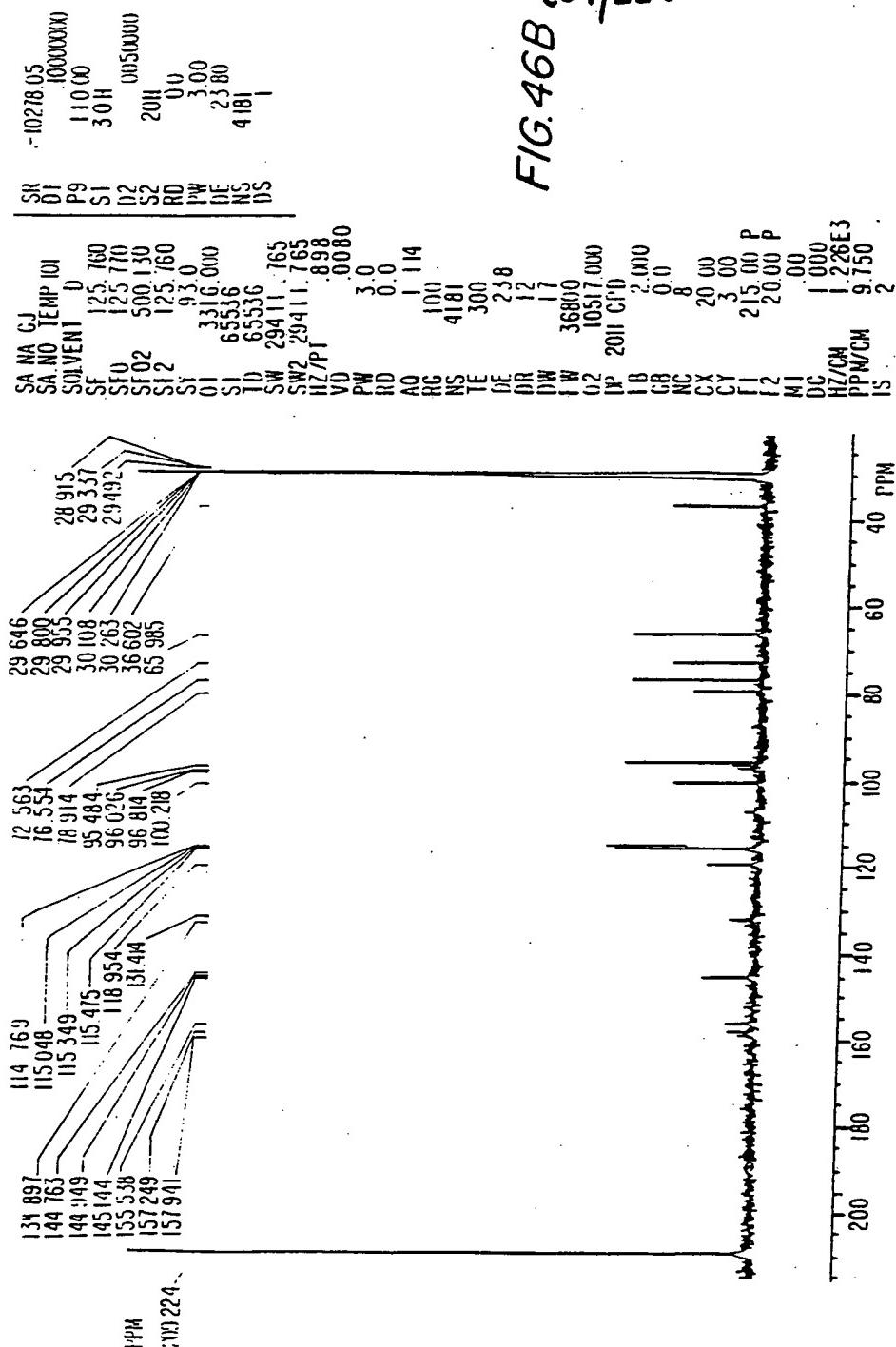
SUBSTITUTE SHEET (RULE 26)

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B2 DIMER			
¹ H	CHEMICAL SHIFT (ppm)	¹³ C	CHEMICAL SHIFT (ppm)
B4	2.69 2.83	B4 T4	28.92 36.60
T4	4.63	B3	65.99
B3	4.29	T3	72.56
T3	3.85	T2	76.55
T2	4.99	B2	78.91
B2	4.92	T6 OR 8	95.48
B6	5.92	B6	96.03
T6 OR 8	5.91	T6 OR 8	96.81
T6 OR 8	5.98		100.22
B2'	7.12	B2'	114.77
T2'	6.92	T2'	115.05
T5	6.68	T5'	115.35
B5	6.70	B5'	115.48
T6'	6.87	T6'	118.95
B6'	6.58	B6'	
		T1'	131.41 131.90

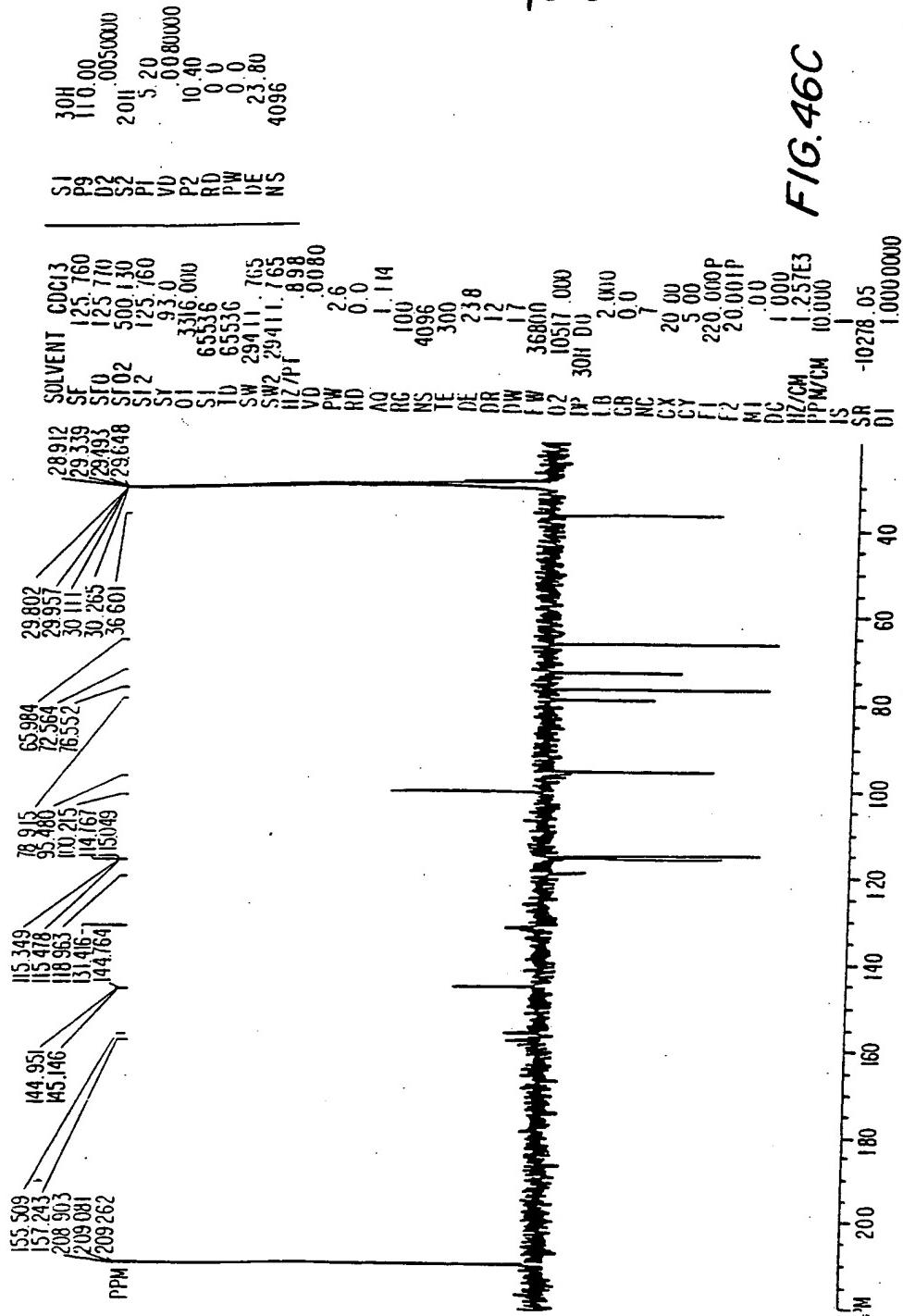
FIG. 46A

FIG. 46B 201/222



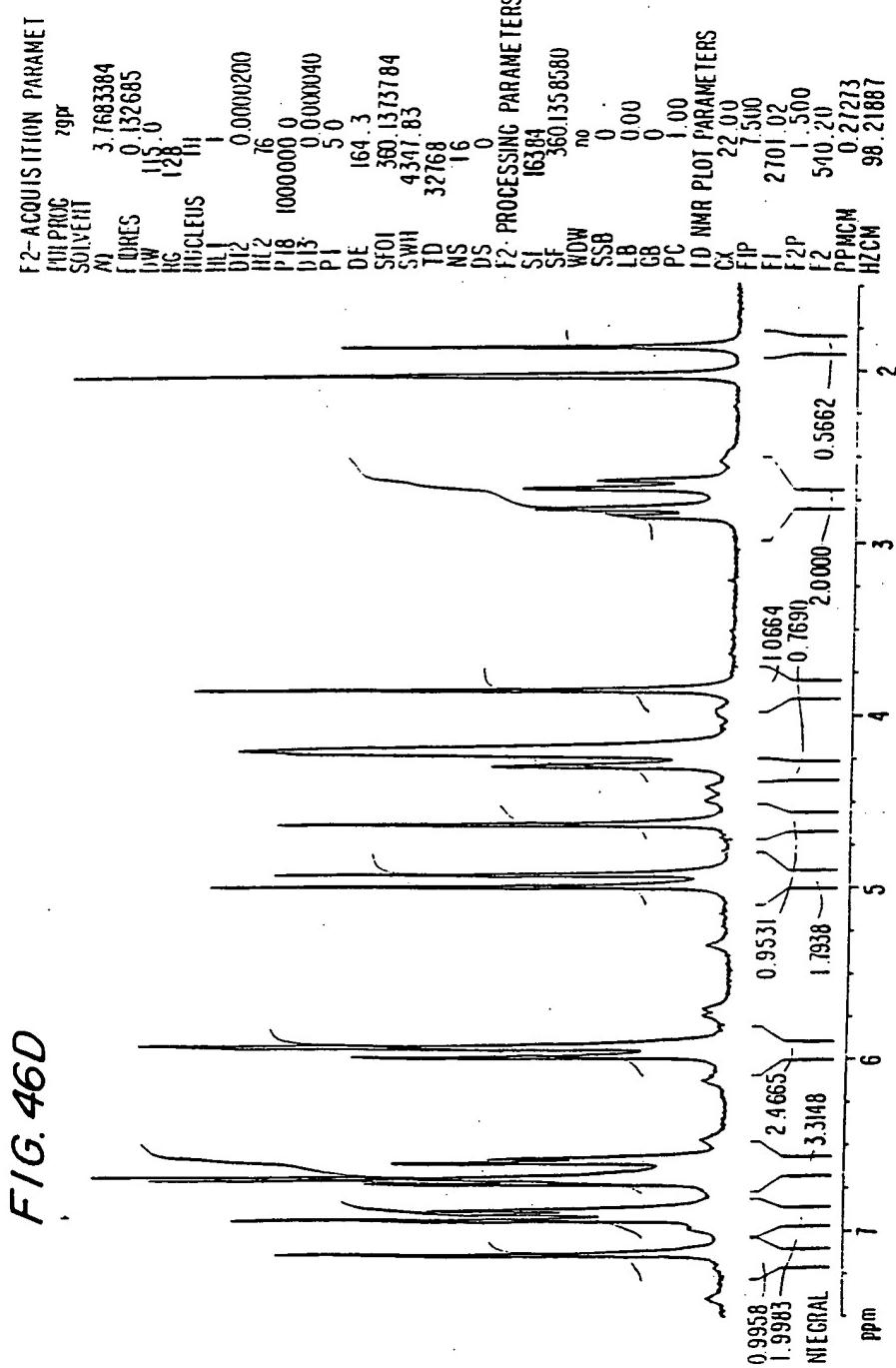
SUBSTITUTE SHEET (RULE 26)

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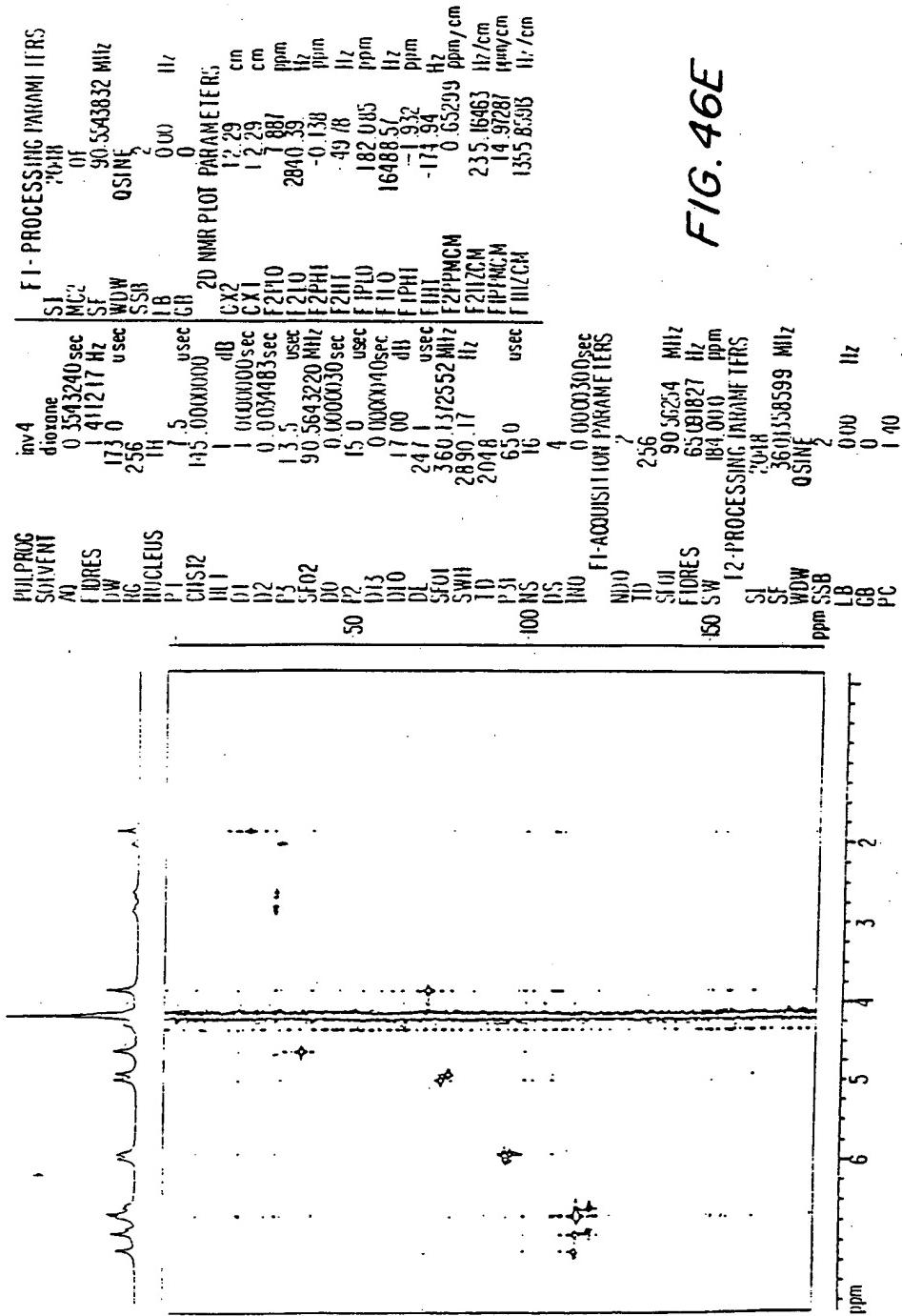


SUBSTITUTE SHEET (RULE 26)

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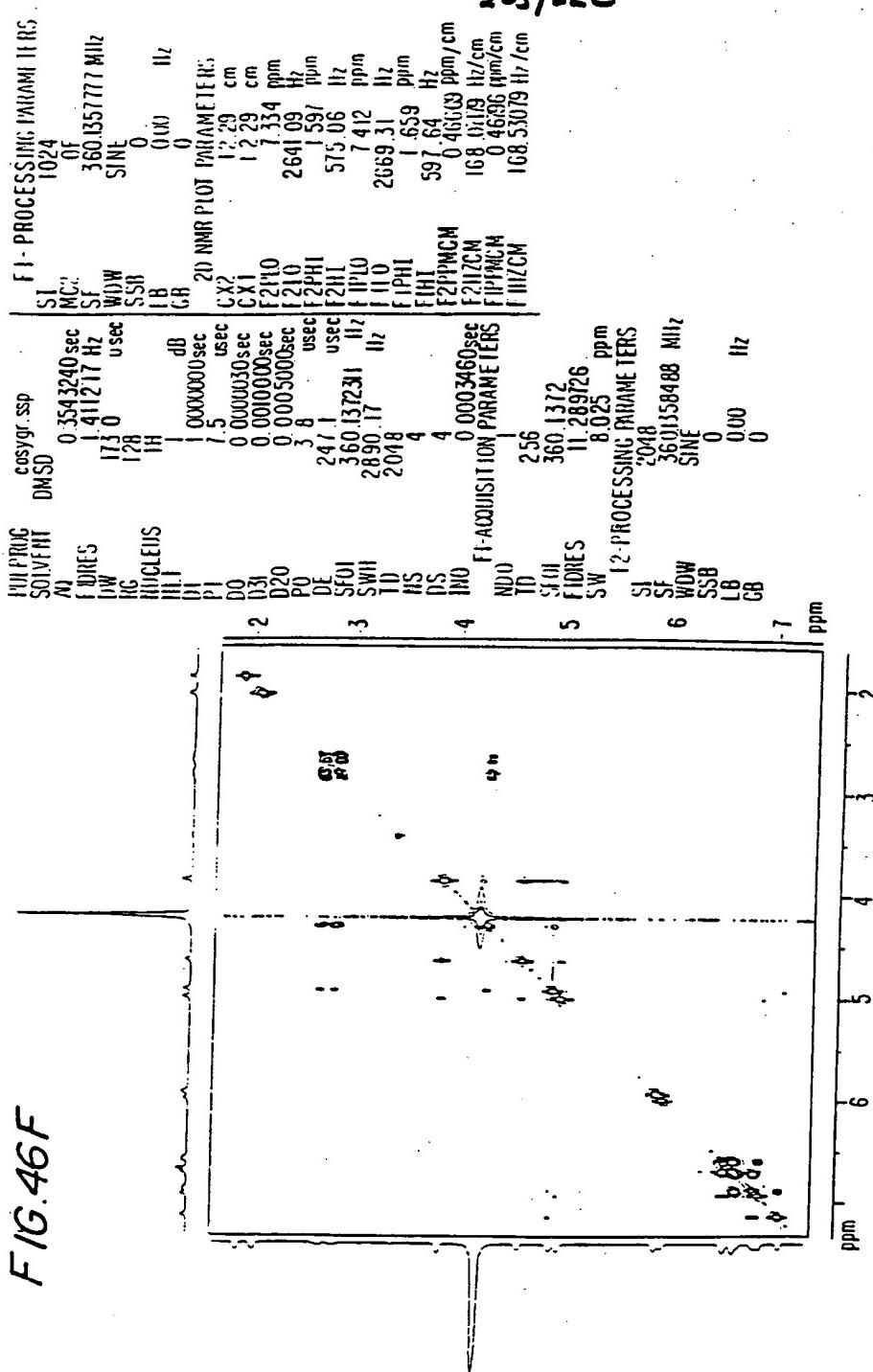


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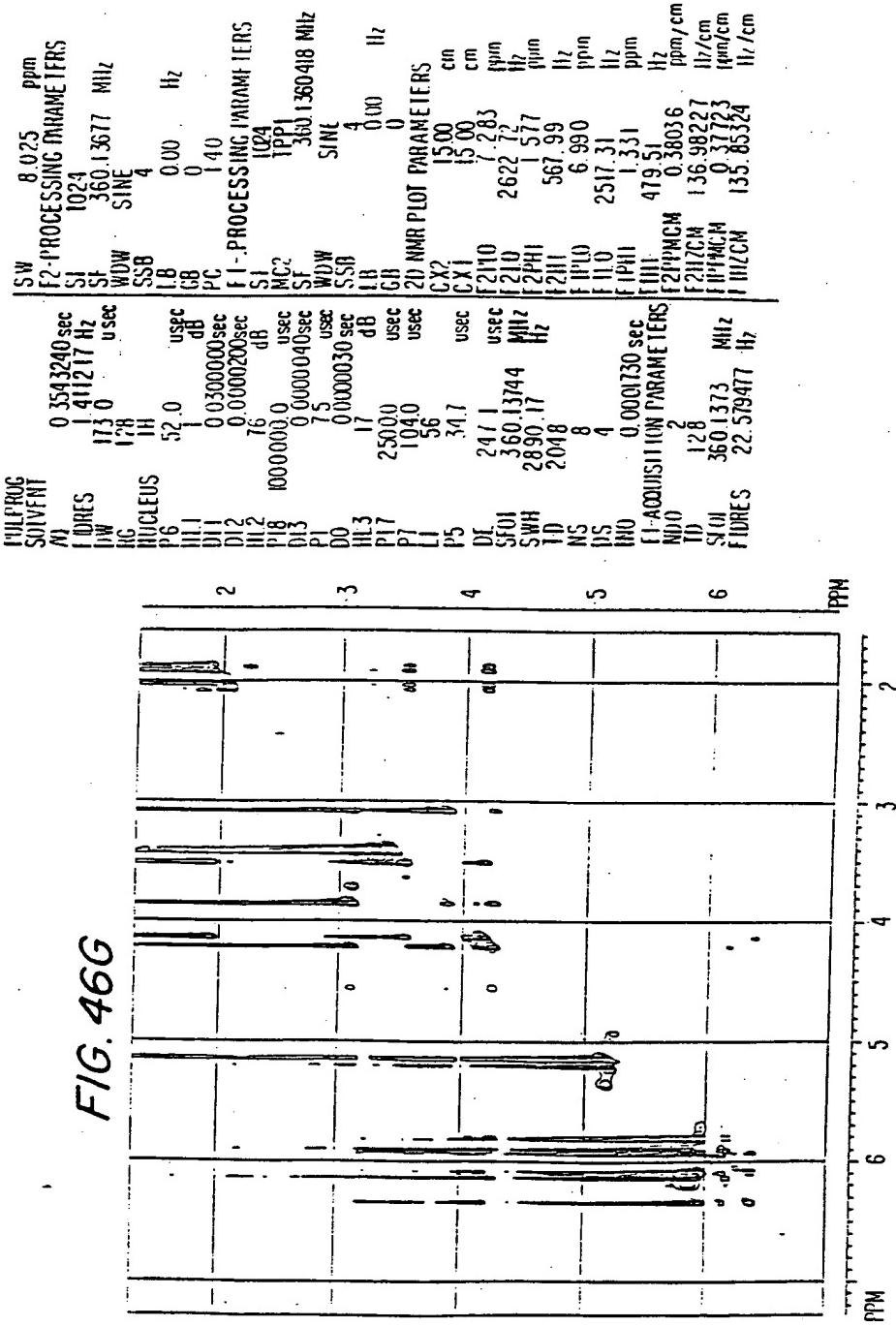


SUBSTITUTE SHEET (RULE 26)

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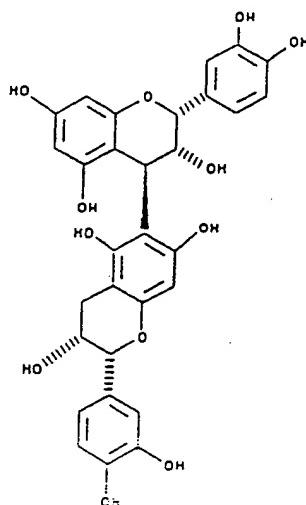


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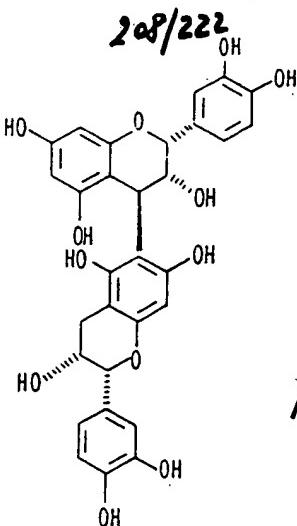
SUBSTITUTE SHEET (RULE 26)

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B5 DIMER			
¹ H	CHEMICAL SHIFT (ppm)	¹³ C	CHEMICAL SHIFT (ppm)
B4	2.585 7.745	B4 T4	29.04 37.03
T3	3.99	B3	66.49
B3	4.13	T3	71.73
T4	4.54	T2	76.69
B2	4.77	B2	78.98
T2	4.90	B6 + 8	95.49
T6+	6.02		96.11
T8	6.055	T6 + 8	96.32
B8	6.04		100.18
T6'	6.64	T2' + 5'	115.01
T5'	6.74	B2' + 5'	115.38
B5'	6.75	B6	118.90
B6'	6.78	T6	118.98
T2'	6.95	B1'	131.62

FIG. 46 H

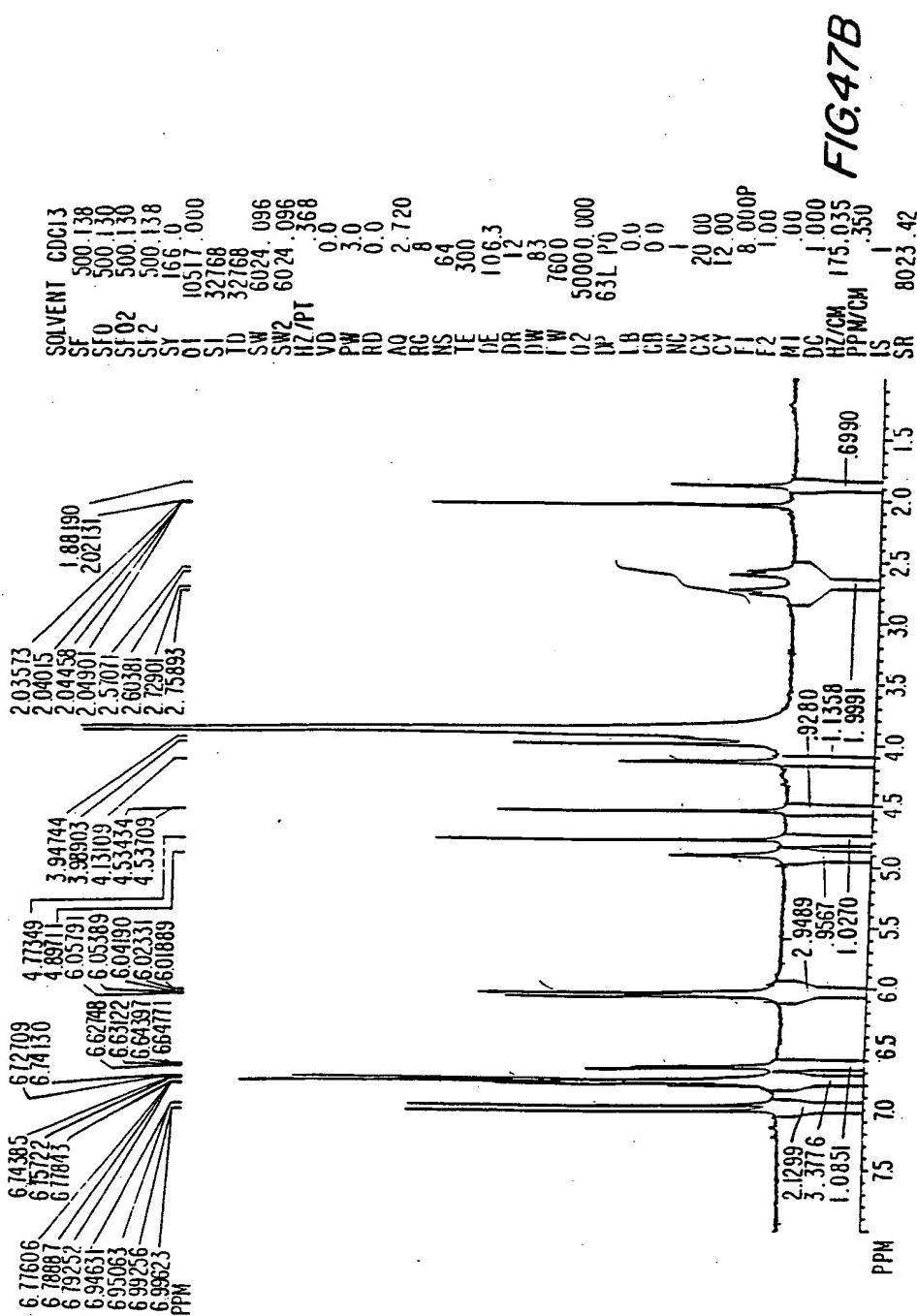
*FIG. 47A*

B5 DIMER			
^1H	CHEMICAL SHIFT (ppm)	^{13}C	CHEMICAL SHIFT (ppm)
B4	2.585 7.745	B4 T4	29.04 37.03
T3	3.99	B3	66.49
B3	4.13	T3	71.73
T4	4.54	T2	76.69
B2	4.77	B2	78.98
T2	4.90	B6+8	95.49
T6+	6.02		96.11
T8	6.055	T6+8	96.32
B8	6.04		100.18
T6'	6.64	T2+5	115.01
T5'	6.74	B2'+5'	115.38
B5'	6.75	B6	118.90
B6'	6.78	T6	118.98
T2'	6.95	B1'	131.62
B2'	6.99	T1'	131.72
		B & T	145.00
		3' + 4'	145.04 145.04 145.12 145.20
		B + T 5, 7 + 8a	154.73 155.50 157.44

SUBSTITUTE SHEET (RULE 26)

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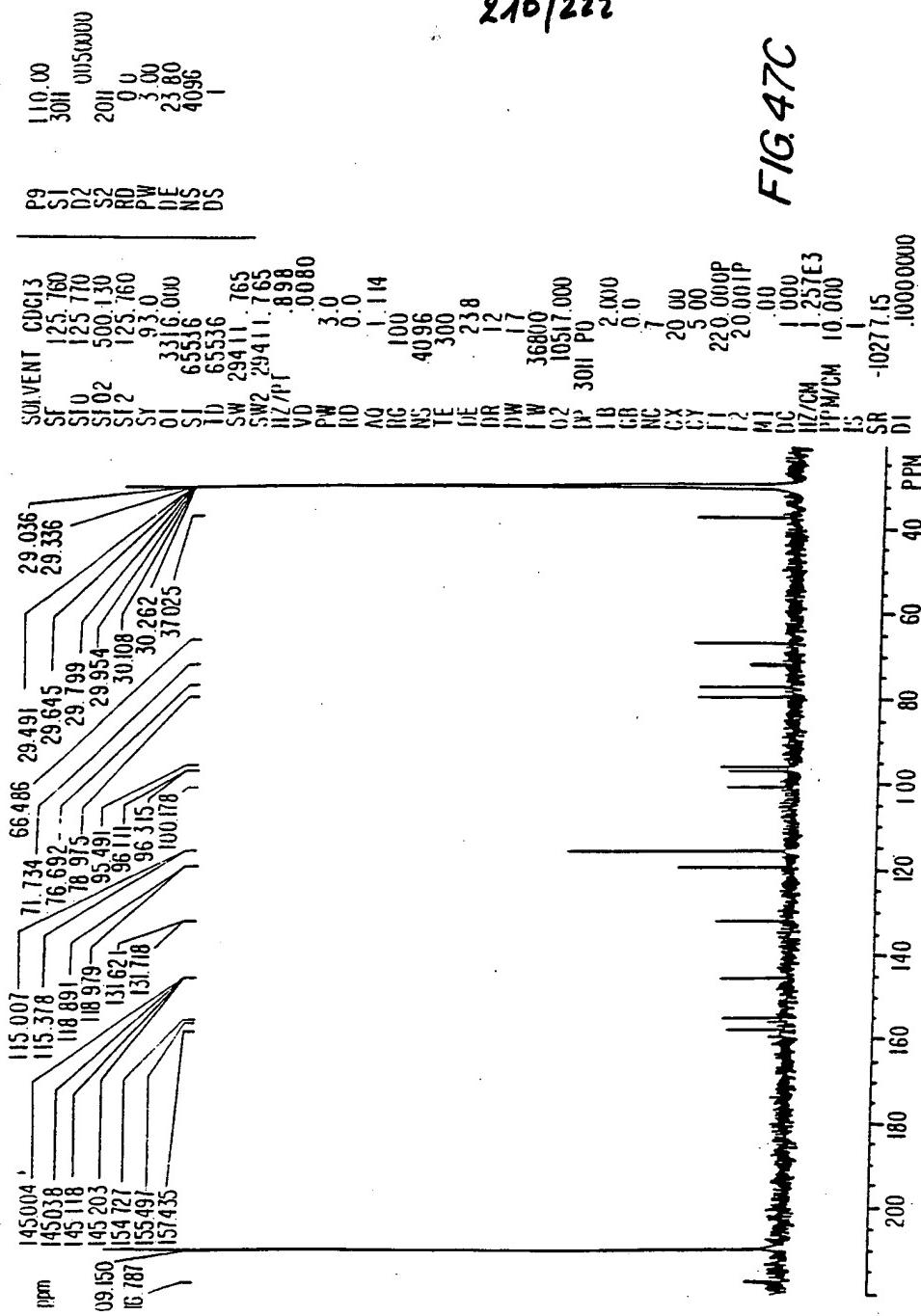
FIG. 47B



SUBSTITUTE SHEET (RULE 26)

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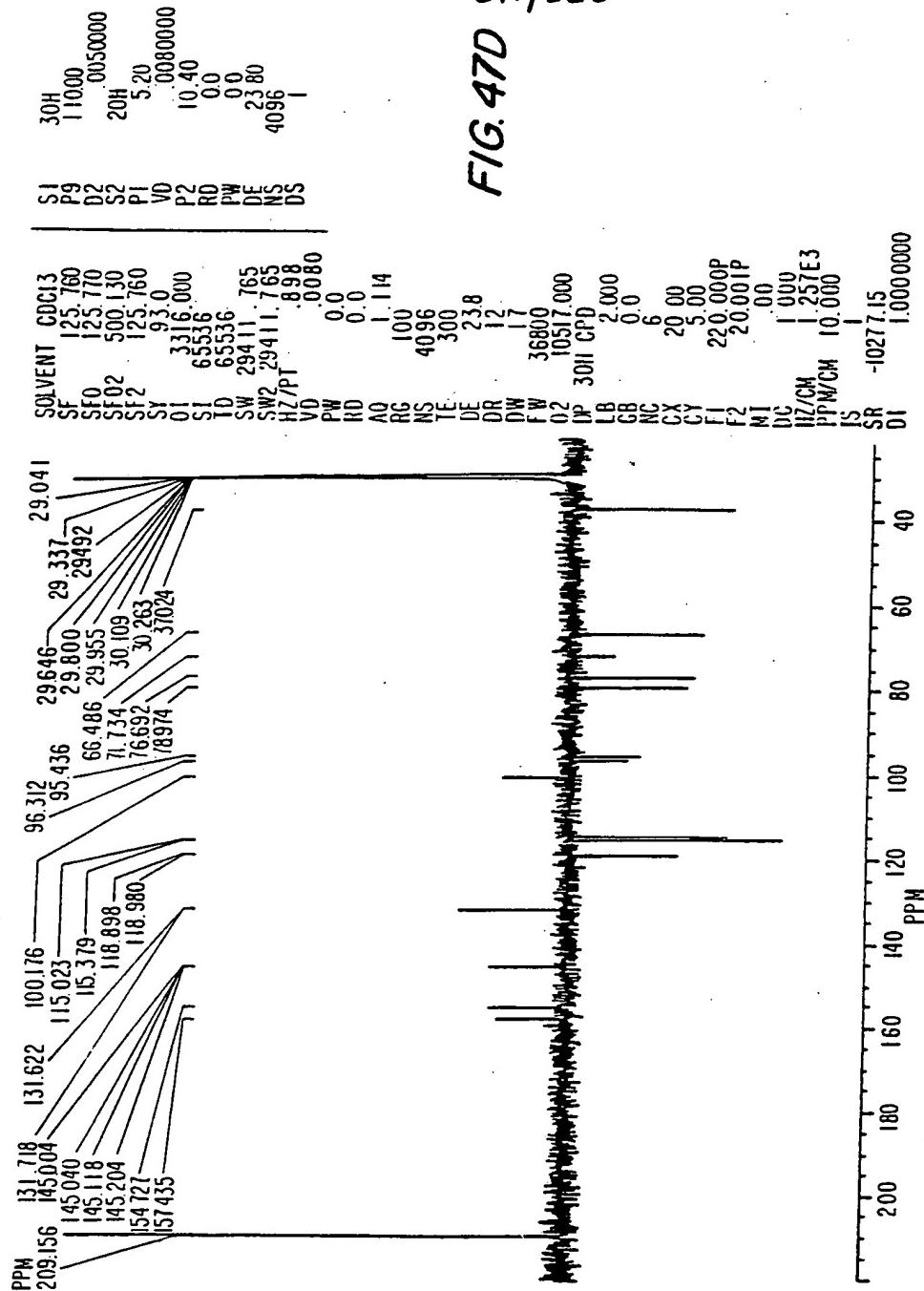
FIG. 47C



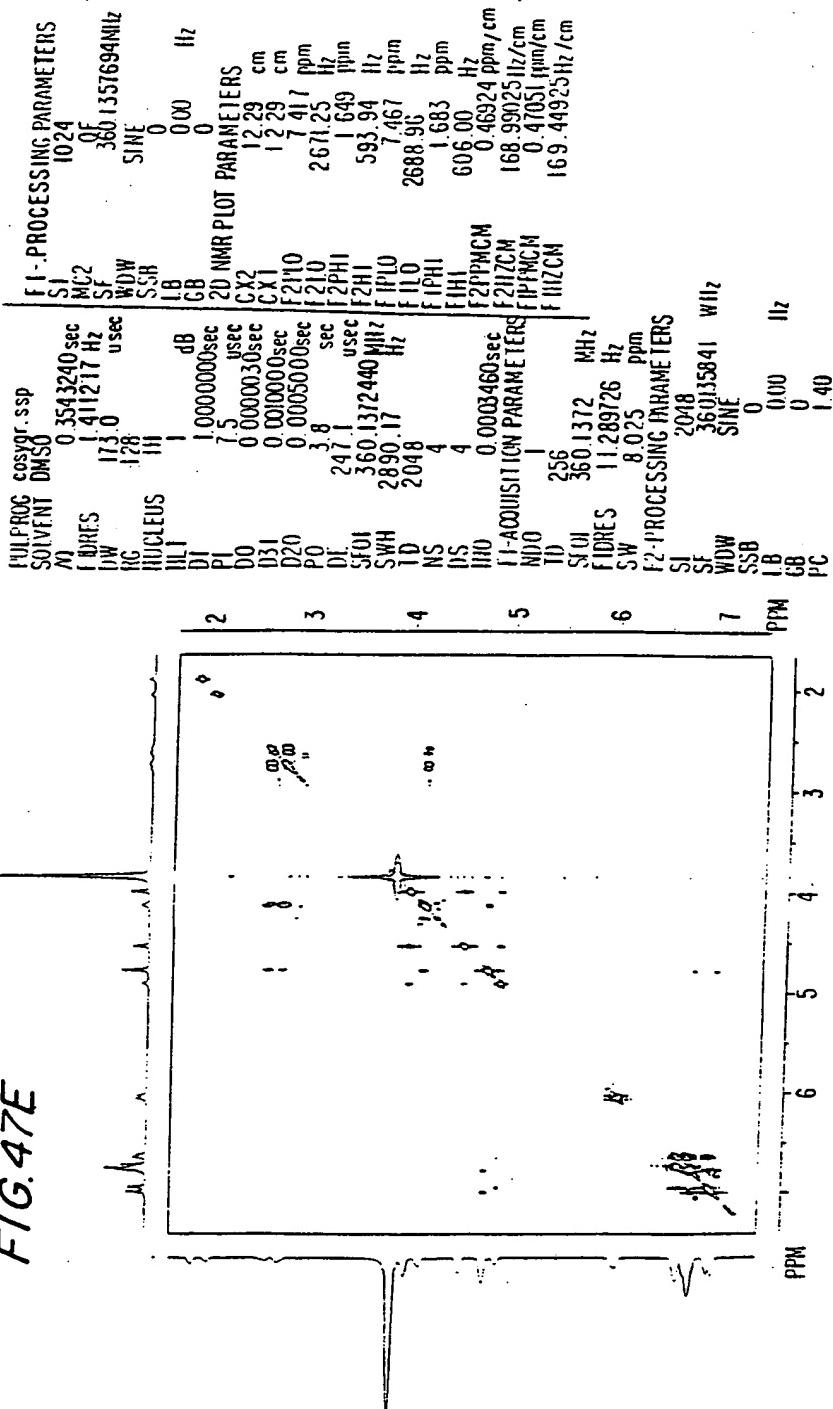
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FIG. 470



SUBSTITUTE SHEET (RULE 26)



SUBSTITUTE SHEET (RULE 26)

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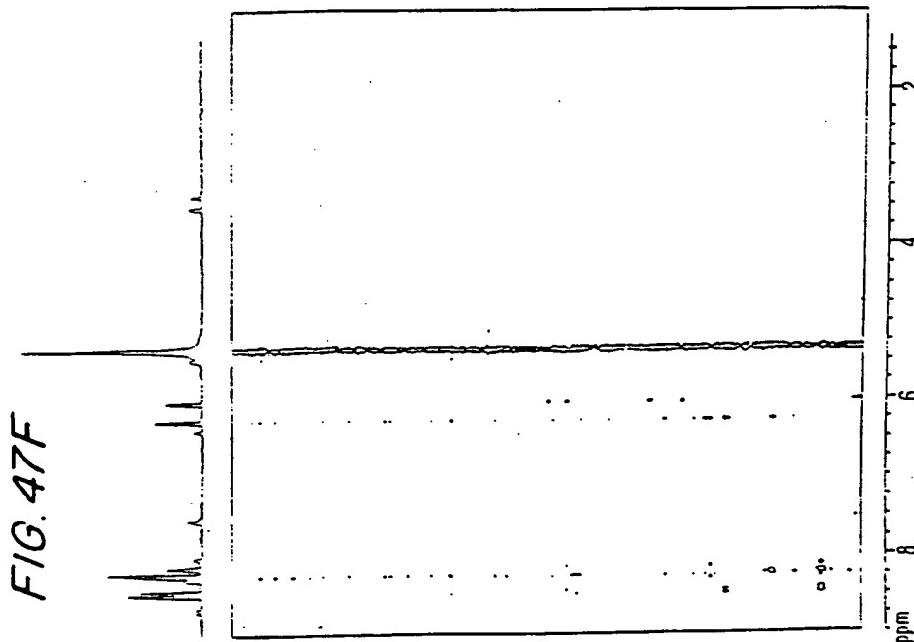
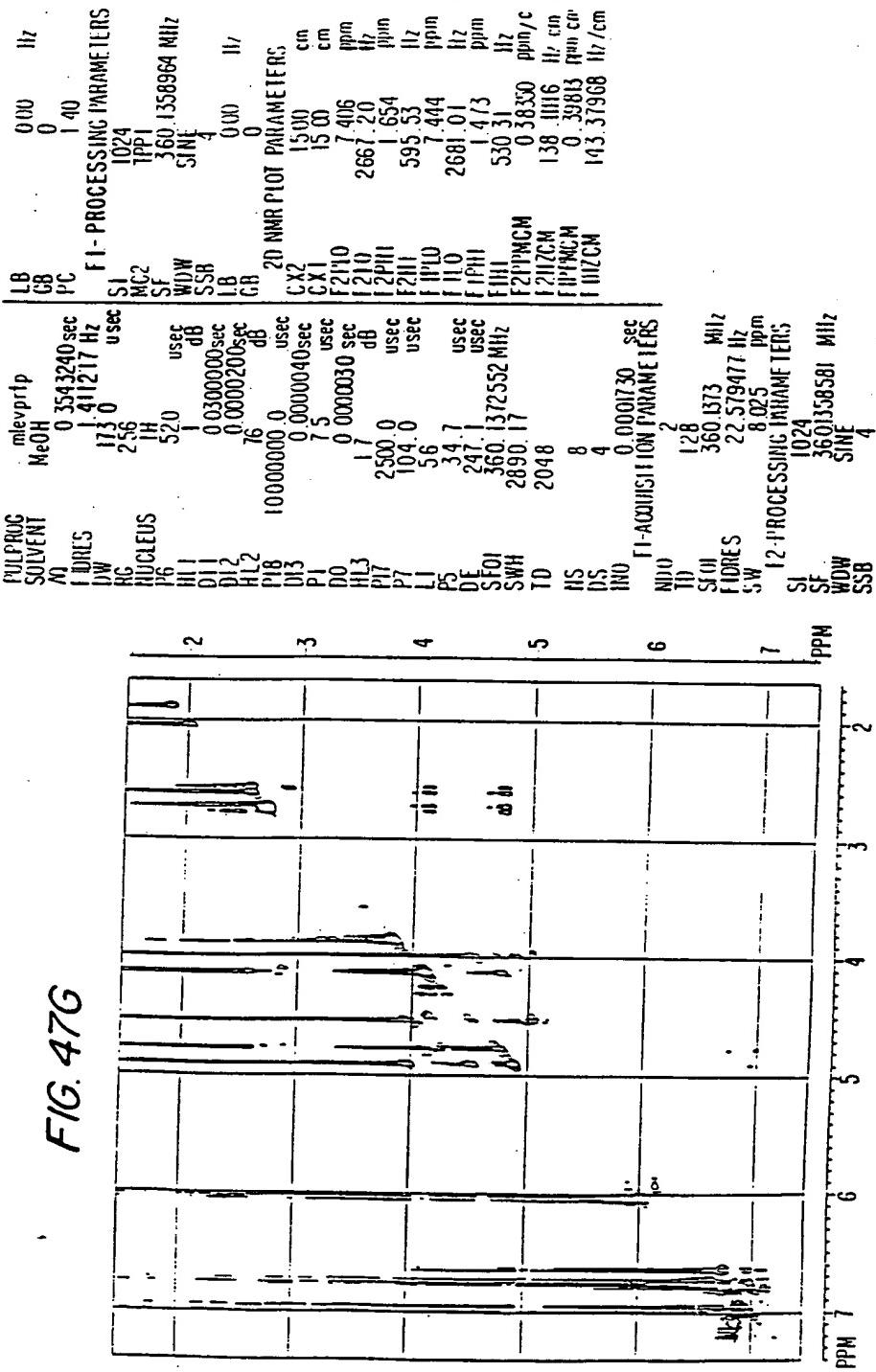


FIG. 47F

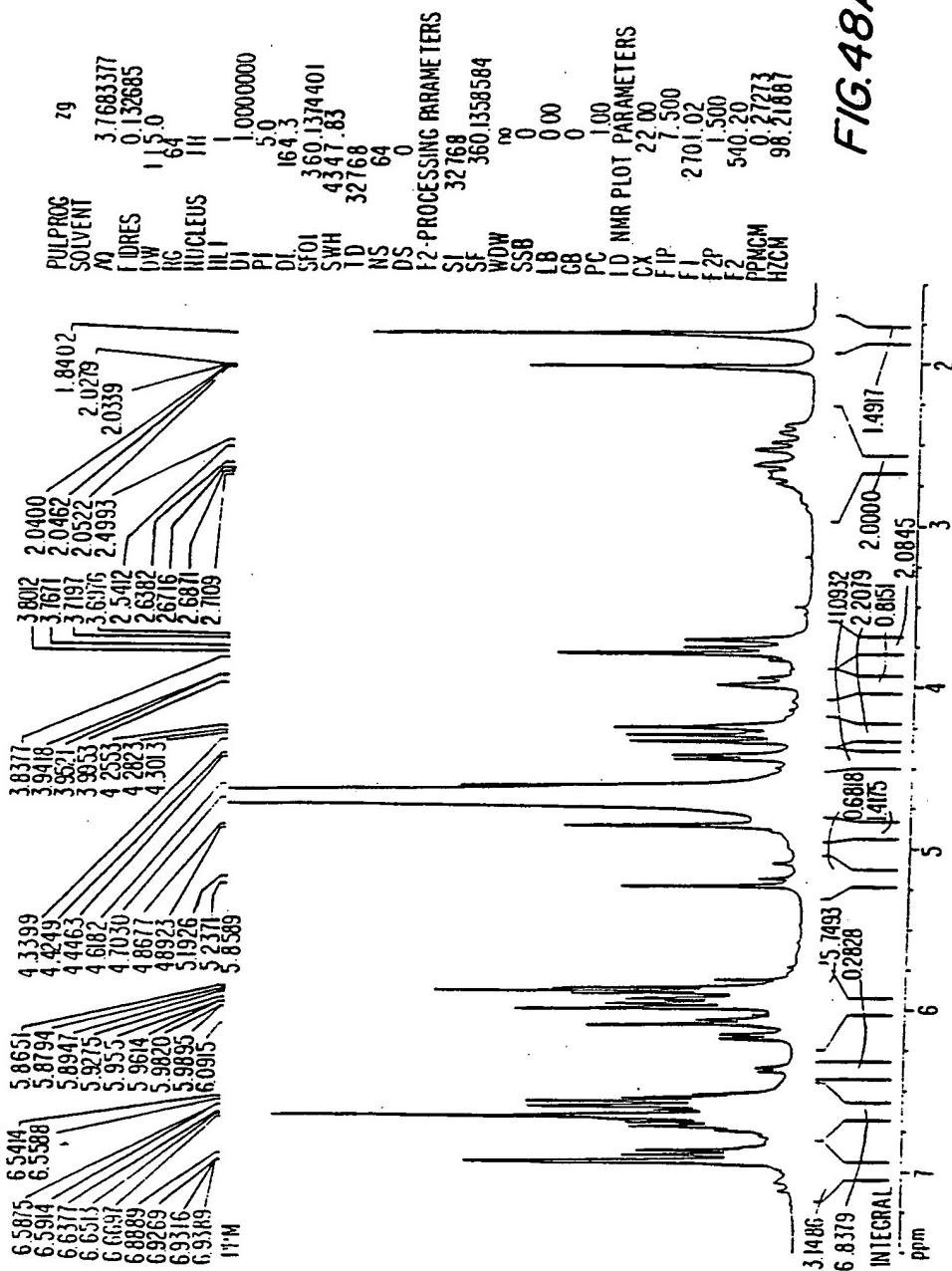
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SUBSTITUTE SHEET (RULE 26)

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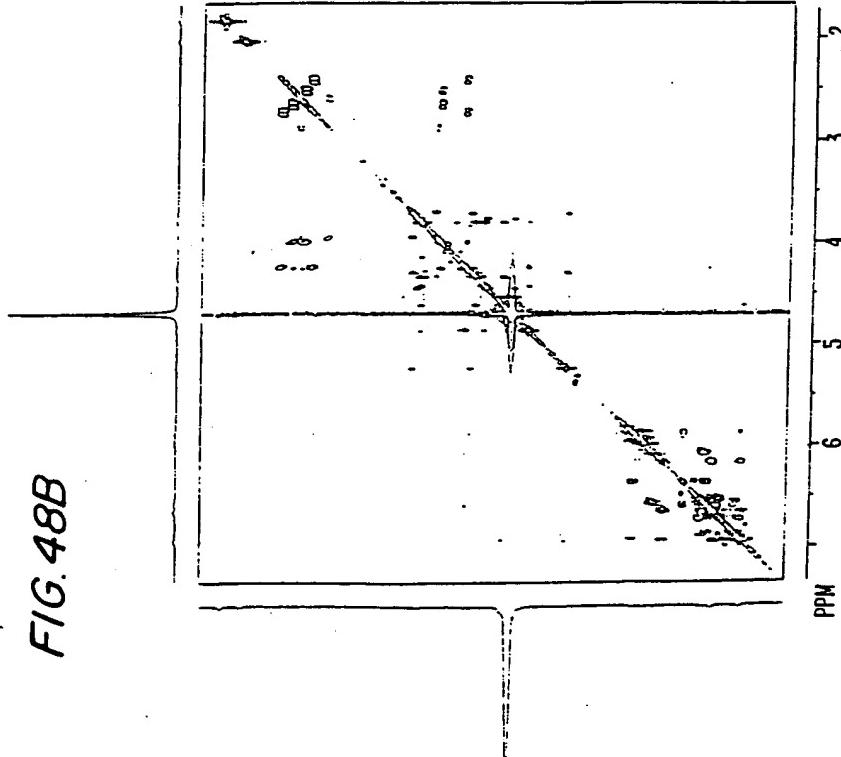


SUBSTITUTE SHEET (RULE 26)

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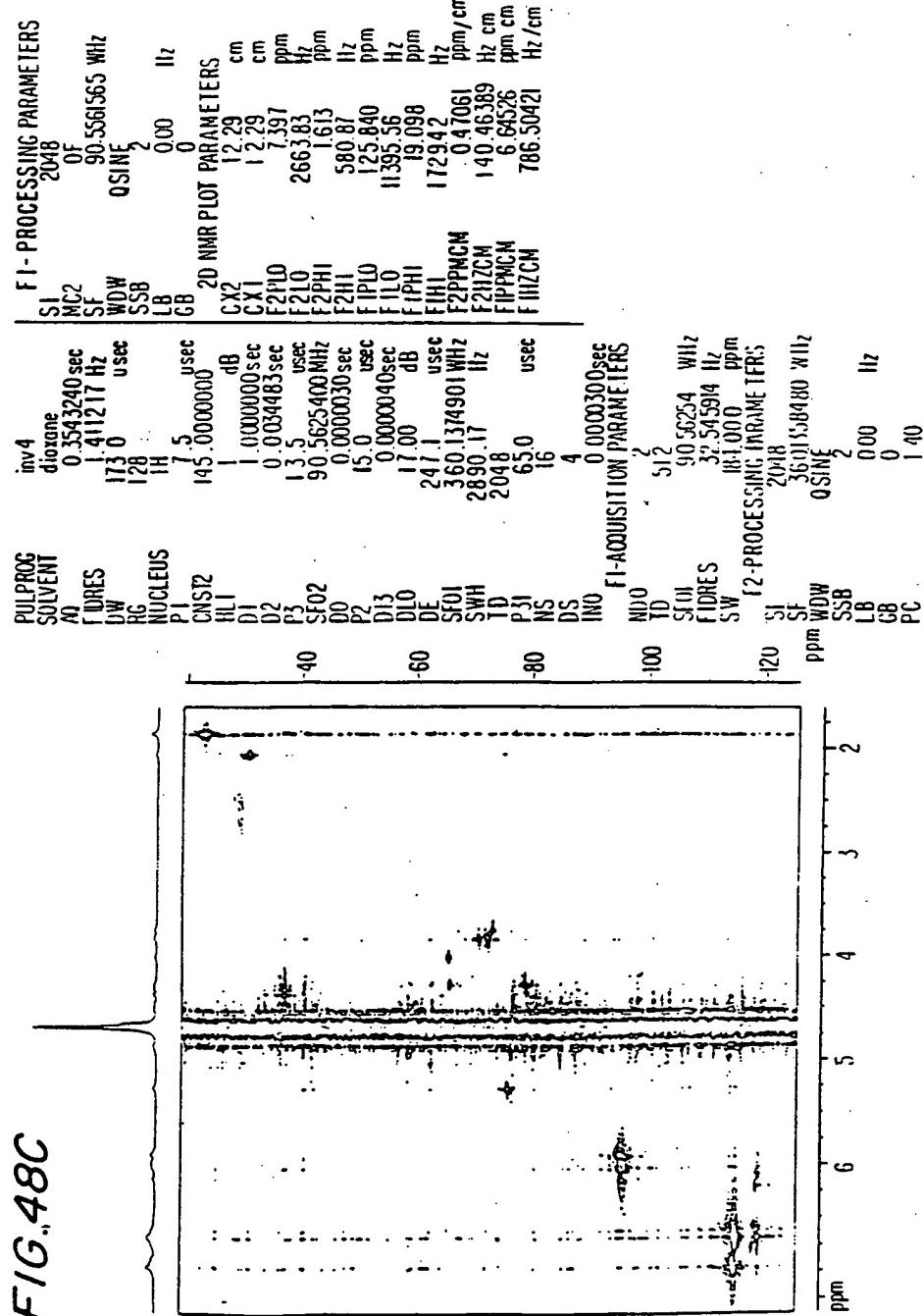
F1-PROCESSING PARAMETERS	
SI	1624
MC2	360
SF	1355236 MHz
WDW	SINE
SSB	0
LB	0.00
GB	0
2D NMR PLOT PARAMETERS	
C12	12.29 cm
CX1	1.229 cm
F2110	7.551 ppm
F2PH1	2641.551 Hz
F2H1	604.09 Hz
F1D	7.360 ppm
F1H1	590.06 ppm
F2PPMCM	0.46159 ppm/cm
F2HZCM	166.23495 Hz/cm
F1PHCM	0.46541 ppm/cm
F1ZCM	167.6238 Hz/cm
F1-AQUISITION PARAMETERS	
SI	0.0003460 sec
DS	4
TD	2048
NS	8
SW	360.1372 MHz
ND	1
TD	256
SI	1.289726 Hz
SW	8.025 ppm
ND	1
TD	2048
SI	360.156548 MHz
WDW	SINE
SSB	0
LB	0.00
GB	0
PC	140

FIG. 48B



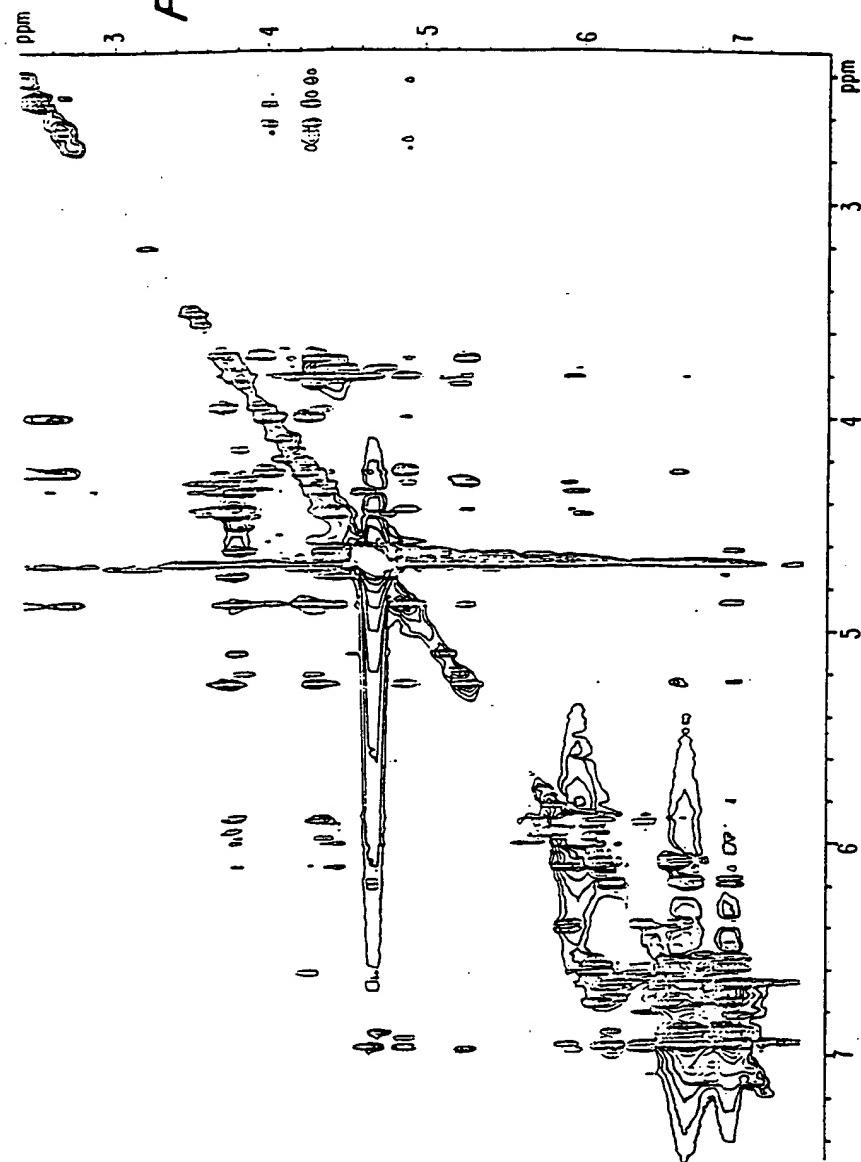
SUBSTITUTE SHEET (RULE 26)

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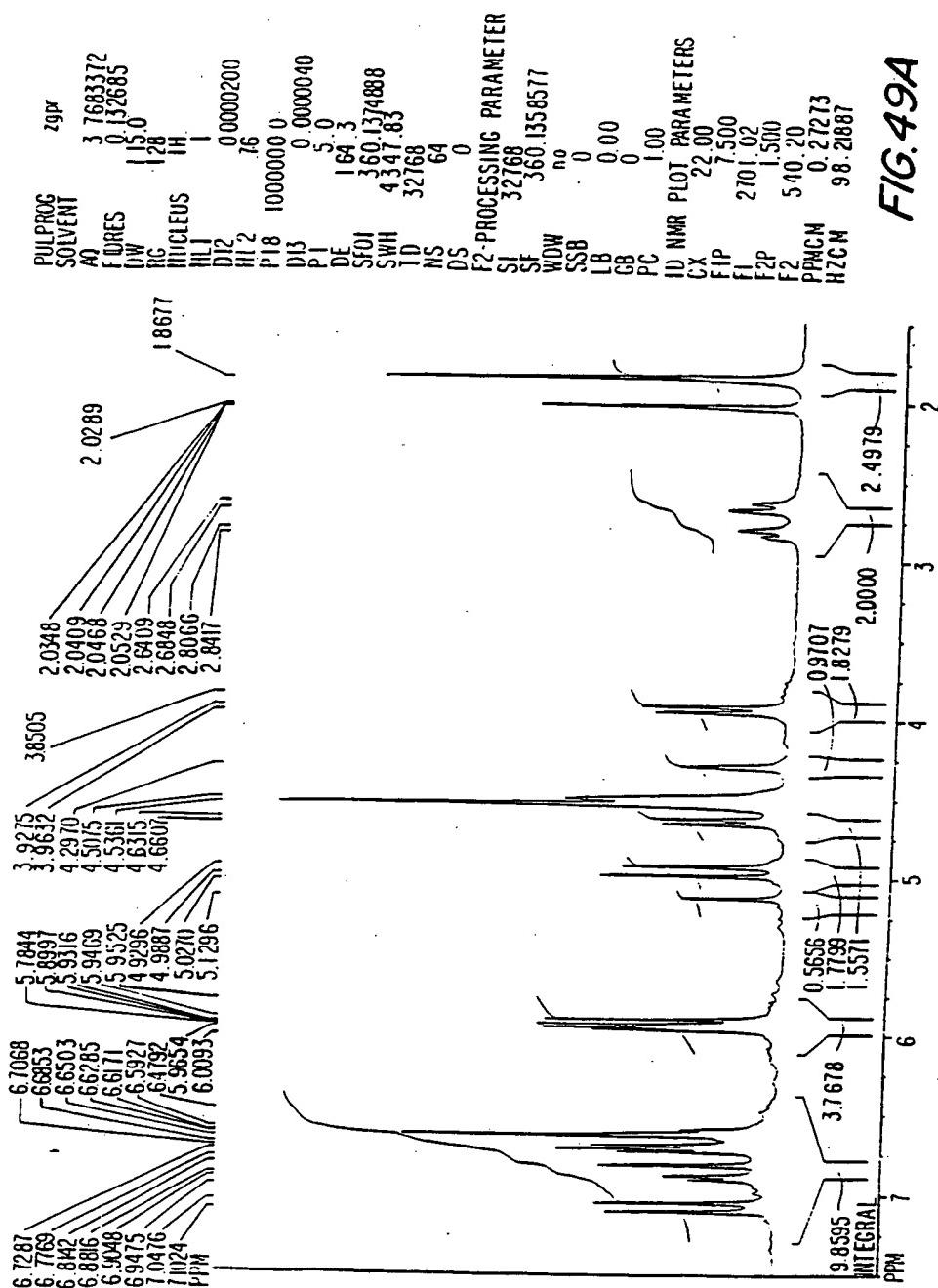
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FIG. 48D



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SUBSTITUTE SHEET (RULE 26)

FIG. 494

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F1-PROCESSING: INNAMT 11CS

1	PROG	cosine ssb	SI	1024
2	SOLVENT	DMSO	SI	OF
3	TD	0	MC ₂	360
4	SW	3543240 sec	SI	1355236 MHz
5	FOURIER	411217 Hz	SW	0
6	DATA	1130 usec	SSB	0
7	INC	128	WID	0
8	NUCLESUS	111	SWF	0
9	DT	000000000 sec	SWI	0
10	P1	000000000 sec	SWR	0
11	DI	000000000 sec	SWR	0
12	D0	000000000 sec	SWR	0
13	D31	000000000 sec	SWR	0
14	D20	000000000 sec	SWR	0
15	P0	000050000 sec	SWR	0
16	P1	000000000 sec	SWR	0
17	DL	291 sec	SWR	0
18	SWF1	360.134998 MHz	SWR	0
19	SWH	289017 Hz	SWR	0
20	ID	2010	SWR	0
21	NS	8	SWR	0
22	DS	4	SWR	0
23	RD	0	SWR	0
24	SW	0000460 sec	SWR	0
25	SWRES	11289126 Hz	SWR	0
26	SWF	8.025 ppm	SWR	0
27	SWF1	200 Hz	SWR	0
28	SWF2	350.1358548 MHz	SWR	0
29	SWF3	0	SWR	0
30	SWF4	0	SWR	0
31	SWF5	0	SWR	0
32	SWF6	0	SWR	0
33	SWF7	0	SWR	0
34	SWF8	0	SWR	0
35	SWF9	0	SWR	0
36	SWF10	0	SWR	0
37	SWF11	0	SWR	0
38	SWF12	0	SWR	0
39	SWF13	0	SWR	0
40	SWF14	0	SWR	0
41	SWF15	0	SWR	0
42	SWF16	0	SWR	0
43	SWF17	0	SWR	0
44	SWF18	0	SWR	0
45	SWF19	0	SWR	0
46	SWF20	0	SWR	0
47	SWF21	0	SWR	0
48	SWF22	0	SWR	0
49	SWF23	0	SWR	0
50	SWF24	0	SWR	0
51	SWF25	0	SWR	0
52	SWF26	0	SWR	0
53	SWF27	0	SWR	0
54	SWF28	0	SWR	0
55	SWF29	0	SWR	0
56	SWF30	0	SWR	0
57	SWF31	0	SWR	0
58	SWF32	0	SWR	0
59	SWF33	0	SWR	0
60	SWF34	0	SWR	0
61	SWF35	0	SWR	0
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FIG. 49B

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04497

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/65, 31/70; C07D 321/00; C07H 15/24
US CL : 514/25, 100; 536/18.1; 549/200

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols).

U.S. : 514/25, 100; 536/18.1; 549/200

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 3,892,879 (HETZEL et al.) 01 July 1975, column 10, line 38.	1-47

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
O document referring to an oral disclosure, use, exhibition or other means		
P - document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search Date of mailing of the international search report

16 JULY 1996

27 AUG 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Faxsimile No. (703) 305-3230

Authorized officer

ELLI PESELEV

Telephone No. (703) 308-1235

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04497

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/65, 31/70; C07D 321/00; C07H 15/24
US CL :514/25, 100; 536/18.1; 549/200

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/25, 100; 536/18.1; 549/200

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

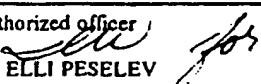
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Special categories of cited documents:		
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search	Date of mailing of the international search report
16 JULY 1996	27 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C., 20231	Authorized officer  ELLI PESELEV
Faxsimile No. (703) 305-3230	Telephone No. (703) 308-1235